THE UNIVERSITY *of* EDINBURGH

THE IN VITRO CULTURE AND TRANSPOSON-MEDIATED GENETIC MODIFICATION OF CHICKEN PRIMORDIAL GERM CELLS

JONI MACDONALD

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DECLARATION

I declare that the work presented in this thesis is my own, except where otherwise stated. All experiments were designed by, myself in collaboration with my supervisors Prof. Helen Sang and Dr. Mike McGrew. No part of this thesis has been, or will be submitted for any other degree, diploma or qualification.

Joni Macdonald
September 2011

ABSTRACT

Primordial germ cells (PGCs) are the embryonic precursors of the germ cell lineage. Segregation of the chicken germ line from somatic cells occurs very early in embryonic development. By day two of incubation chicken PGCs can be isolated from the circulating blood. The *in vitro* culture of chicken PGCs has significant potential as a tool for the investigation of germ cell development and as a cell-based system for the production of genetically modified chickens. The isolation, culture and manipulation of migratory chicken PGCs reported previously have not been independently validated.

Initial attempts to isolate and culture chicken PGCs by reproducing a published protocol proved difficult. Key components of the published culture medium are by their nature variable, including the use of BRL-conditioned medium and animal sera. The protocol also stated that addition of SCF to the culture medium is essential but did not identify the source of SCF used. Several components of the culture conditions were tested including sources and batches of bovine and chicken sera and the growth factors FGF2 and SCF. Chicken PGCs from wild type and GFP-expressing chicken embryos were cultured and several cell lines established, proliferating for more than 100 days in culture. After seventy days in culture a single chicken PGC cell line was shown to retain the potential to develop into functional sperm. This was demonstrated by injection of the cultured chicken PGCs into early chick embryos, which were hatched and produced offspring derived from the injected chicken PGCs.

To understand and produce a more robust system for the isolation and propagation of chicken PGCs three signalling pathways, AKT, MAPK and JAK/STAT, were investigated. When any of these signalling pathways were blocked, using chemical inhibitors, chicken PGC proliferation *in vitro* was significantly inhibited, showing the pathways to be essential for chicken PGC proliferation. Chicken PGCs were treated with individual components of the standard culture medium, FGF2, SCF, animal sera, BRL-conditioned medium, LIF and IGF, and the activation status of the key signalling pathways was assessed by western blot. Individual components of the culture medium induced activation of the AKT and MAPK pathways but not the JAK/STAT pathway. These data increase our understanding of PGC biology and are the first steps towards the development of a feeder- and serum-free medium for the growth of chicken PGCs.

Published methods for the genetic manipulation of chicken PGCs are inefficient. To improve the efficiency of stable transgene integration, transposable element-derived gene transfer vectors were assessed for their ability to transpose into the genome of chicken PGCs. Comparison of Tol2 and piggyBac transposable elements, carrying reporter transgenes, demonstrated that both can be used to genetically-modify chicken cells. The incidence of stable transposition achieved was higher when using the Tol2 transposable element in comparison to the piggyBac element. The genetically-modified chicken PGCs formed functional gametes, demonstrated by injection of genetically modified chicken PGCs into host embryos which were hatched and produced transgenic offspring expressing the reporter gene construct.

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CONTENTS

Chapter 5: Genetic manipulation of cultured chicken primordial germ cells

Chapter 6: General discussion

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

µl	microlitre
AKT	protein kinase B
ALV	Avian leukosis virus
BMP	Bone morphogenic protein
BRL	Bovine rat liver cell
CEF	Chicken embryonic fibroblast
CVH	chicken vasa homolog
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecos modified eagle medium
EDTA	Ethylenediaminetetraacetic acid
EG&K	Eyal-Giladi and Kochav (1976)
ERK	Extracellular related kinase
ES cell	embryonic stem
FBS	Foetal bovine serum
FCS	Foetal calf serum
FGF2	Fibroblast growth factor 2
FKHR	Forkhead (Drosophila) homolog 1
FRS2	Fibroblast growth factor receptor substrate 2
Gab	Grb2-associated binding protein
Grb2	Growth receptor bound protein 2
GSK3beta	Glycogen synthase kinase 3 beta
HH	Hamburger and Hamilton (1951)
IGF1	Insulin-like growth factor 1
JAK	Janus kinase
JNK	Jun N-terminal kinase
Klf4	Kruppel-like factor
LIF	Leukaemia inhibitory factor
MAPK	Mitogen activated protein kinase
MEK	MAPK/ERK kinase
ml	millilitre
mTOR	Mammalian target of rapamycin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK	Phosphatidylinositol-3-phosphate dependent kinase
PEC	Paired end complex
PGC	Primordial germ cell
PI3K	Phosphoinositol 3 kinase
PSM	Presomitic mesoder
PtdIns	Phosphatidyinositol
PTEN	Phosphatase/tensin homolog
RA	Retinoic acid
REV	Reticuloendothelial virus

ROCK	Rho Kinase
RT-PCR	Reverse transcriptase PCR
SCF	Stem cell factor
SDF1	Stromal derived factor 1
SH2	Src Homology 2
SHC	Src homology 2 domain-containing
SOCS	Suppressor of cytokine signalling
SOS	Son of Sevenless
Sox	Sex determining region Y-box
STAT	Signal ransducers and activators
STO	Sandoz inbred mouse-derived thioguanine- and ouabain- resistant

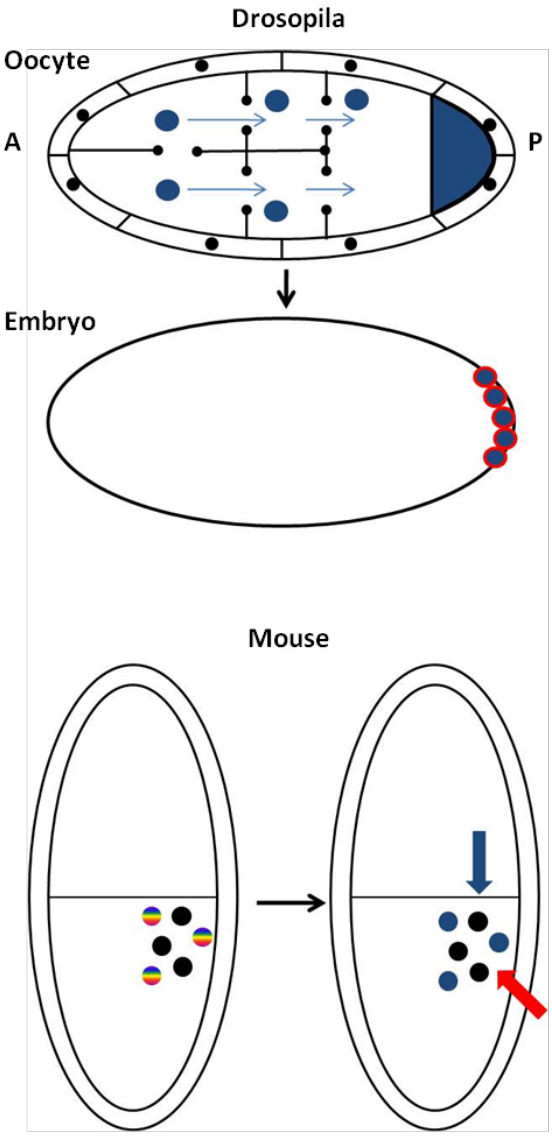
CHAPTER 1: GENERAL INTRODUCTION

The key to the survival of a species is its ability to reproduce. In sexually reproducing organisms this requires the formation of gametes to generate the next generation. The gametes derive from primordial germ cells (PGCs). PGCs are the precursors of the germ cell lineage, differentiating to form oocytes in the ovary, and spermatogonia in the testis. They arise early in embryonic development prior to sexual determination, in an extra-gonadal region. During embryonic development PGCs migrate towards the region of gonadal development. In chickens segregation of the germ and somatic cell lineages occurs in the epiblast of the stage X embryo EG&K (Eyal-Giladi and Kochav, 1976) in contrast to mammals where PGCs are specified later in development at the start of gastrulation (Petitte *et al*. 1997). The production of an efficient culture method for the propagation of chicken PGCs *in vitro* will provide a useful system for the study of PGC biology. The chicken PGC's ability to form functional gametes after propagation in culture is useful for the development of a cell-based system for the genetic modification of the chicken genome and the production of transgenic birds. This is a valuable tool with applications in both research and industry (McGrew *et al*. 2004, Sang 2006). Currently no other cell-based method exists for the production of transgenic birds. Although chicken embryonic stem (ES) cells can be isolated they have not been shown to contribute to the germline after short periods in culture (Pain *et al*. 1996; Petitte *et al*. 2004; van de Lavoir *et al*. 2006;). To successfully propagate avian PGCs it is useful to have an understanding of the mechanisms by which they arise and the genetic pathways that regulate germ cell survival, proliferation and migration in the early embryo.

1.1 THE ORIGIN OF PRIMORDIAL GERM CELLS, PREFORMATION OR EPIGENESIS

The mechanism for PGC specification is not conserved between chordates as it is for somatic tissue specification. In the chordates PGCs originate via one of two developmental mechanisms, epigenesis or preformation (Extavour and Akam, 2003;

Johnson *et al*. 2003; Crother *et al*. 2007) (Figure 1.1). Epigenesis is the de-differentiation of pluripotent cells to form PGCs, induced by signalling from surrounding somatic tissues. In contrast, preformation is the cell autonomous specification of PGCs influenced by maternally derived factors. These maternally derived molecules are referred to as the "germ plasm" In this next section preformation and epigenesis will be discussed and examples of species where the germ cell lineage is formed via each of these methods given.



Preformation:
During oogenesis RNAs and proteins (blue) are synthesised by nurse cells and via cytoplasmic bridges are transported to (blue arrows) and become localised at the posterior end (P) of the oocyte. In the initial stages of embryogenesis the germ plasm is inherited by cells, it is only cells that inherit the germ plasm that will develop into the PGCs (red/blue).

Epigenesis:
In the absence of maternally deposited germ plasm PGC determination takes place after the segregation of embryonic and extraembryonic somatic tissues. When these tissues express inductive signals cells primed (multi-colour) to become germ cells differentiate into PGCs (blue). Inductive signals come from the endoderm (red arrow) and the ectoderm (blue arrow)

Figure 1.1. The two modes of germline segregation. (a) Preformation and (b) Epigenesis. Adapted from Extavour and Akam 2003.

1.1.1 Preformation

Preformation of the germline is associated with animals where a germ plasm has been identified in cells of the oocyte or early embryo. The germ plasm is a maternally inherited region of cytoplasm that contains RNA and protein rich granules. The characteristics of the germ plasm are well conserved between species in which the germline arises via preformation (Czolowska, 1972). Species in which germline determination via preformation has been investigated include *Drosophila melanogaster* (*D.melanogaster*), *Caenorhabditis elegans* (*C.elegans*), *Xenopus laevis* (*X. laevis*) and zebrafish (*Danio rerio*). In the different species the germ plasm is referred to by a different name, including pole plasm (*Drosophila*), P-granules (*C elegans*) and germinal granules (*Xenopus*).

The most comprehensive evidence for preformation as a mechanism of germline segregation has been identified in *Drosophila melanogaster* (reviewed by Extavour and Akam, 2003). In *Drosophila,* the germline is specified by the formation of four or five pole cells at the posterior end of the blastoderm. These pole cells are the progenitors that give rise to all the cells of the germ cell lineage (Huettner, 1923). Understanding of the origins of the germline in *Drosophila* was further elucidated by series of one and two stage transplantation experiments (Illmensee and Mahowald 1974; Mahowald *et al*. 1976). It was demonstrated that transplanted sections of posterior polar plasm would induce PGC formation at ectopic sites. This confirmed the ability of the polar plasm to determine the germ cell lineage. Ephrussi and Lehmann (1992) validated these finding by demonstrating that mislocalisation of the maternally derived germline specific RNA *osk* could induce PGC formation in ectopic sites. O*sk* has been shown to be essential for the formation of the pole plasm. Pole plasm has also been confirmed to form in the oocyte prior to fertilization (reviewed by Mahowald 2001). All these data conclude PGC formation and germ cell specification in *Drosophila* is mediated by the maternal determinants located within the pole plasm.

In amphibians, the germline forms via preformation and the presence of a "germ plasm" was first evidenced by Bounoure in 1939 (reviewed in Extavour and Akam 2003). This was the first evidence that preformation occurred in a vertebrate. In another vertebrate, *Xenopus laevis* the vegetal plasm localizes to the vegetal sub-cortex of the oocyte. During oogenesis a mitochondrial rich cytoplasm is synthesized within the vegetal plasm. The cytoplasm often referred to as the mitochondrial cloud associates with RNA, electron dense granules and a host of specific proteins. PGC arise post fertilization from a few cells in which aggregates of vegetal plasm have been segregated (Whitington and Dixon 1975). To confirm germ cell determinants are present in the vegetal pole, ultraviolet irradiation (Tanabe and Kotani 1974; Züst and Dixon 1975) or physical elimination (Buehr and Blackler 1970) was used to compromise or remove the germinal cytoplasm. This resulted in production of sterile frogs thus confirming the presence of germ cell determinants within the vegetal pole cells. Further validation of the presence of germ cell determinants was shown when fertility was restored to sterilized embryos by injecting purified fractions of the vegetal plasm into single somatic blastomeres isolated from 32-cell embryos (Ikenishi *et al*. 1986).

Elucidating PGC specification in *Danio rerio* (zebrafish) was made possible by the identification of the zebrafish homolog of the germ cell specific *vasa* gene (Olsen *et al*. 1997; Yoon *et al*. 1997). Yoon *et al*. (1997) reported that *vasa* mRNA is detectable immediately after fertilization of the oocyte indicating that it is maternally provided. By the 32-cell stage of embryonic development the *vasa* mRNA becomes segregated to just four cells, these cells are the precursors to the PGCs. Braat el al (1999) showed that these cells go on to form the PGCs and eventually the gametes by tracing *vasa* expression in zebrafish. Further cell lineage studies were carried out that demonstrated that although preformation is the mechanism involved in germline segregation in zebrafish and other teleosts, it may not be applicable to all fish species (Braat *et al*. 1999).

1.1.2 Epigenesis

As mentioned previously the defining characteristic of epigenesis is the absence of germ plasm. There was much debate as to the origin of PGCs in mammals (Heys 1931; Everett 1945) until alkaline phosphatase activity was identified as a marker for mouse germ cells (Chiquoine 1954). Alkaline phosphatase was used to identify germ cells in mouse embryos as early as 7-7.5 days post coitum (dpc) (Ginsburg *et al*. 1990) and lineage studies were able to trace the these cells to an even earlier embryological time point, 6.5 dpc (Lawson and Hage 1994). The localisation of putative germ cells posterior to the primitive streak at 6.5 dpc is the earliest identification of mouse PGCs. Even the isolation of the mouse *vasa* homolog did not identify PGCs at an earlier developmental stage (Fujiwara *et al*. 1994; Toyooka *et al*. 2000; Noce *et al*. 2001). The POU domain gene, *oct4,* has also been used as a marker for germ cells in the gonad and is expressed throughout the epiblast of the early gastrula. Expression of *oct4* is not restricted in the epiblast and does not become specified to the PGCs until later stages in development. These observations suggest that the germline in mice is not predetermined but arise via epigenesis (reviewed by Tsang *et al*. 2001). Transplantation of the distal and proximal mouse epiblast cells from the region of PGC formation to other regions within the embryo did not result in the emergence of PGCs. This suggests that inductive signals, such as bone morphogenic proteins (BMPs), may be required for PGC formation in the region posterior to the primitive streak. It was further observed that BMP signalling was required for PGC formation from cells of the proximal epiblast (Lawson *et al*. 1999; Ying *et al*. 2000; Ying and Zhao 2001). PGCs could only be differentiated from cells of the proximal epiblast that already expressed *fragillis* and *stella* (Saitou *et al*. 2002). However, in contradictory findings it had been previously demonstrated that proximal epiblast cells that did not express *fragillis* and *stella* were still able to form PGCs when exposed to BMP signalling (Tam and Zhou 1996). These results clearly support the view that the mouse germline is specified by epigenesis and not maternal determinants.

1.1.3 Preformation or epigenesis: chicken primordial germ cells

As has been described above there are two mechanisms for the determination of the germline in animals. Segregation of the chicken germline is not fully understood and there is some debate as to whether chicken PGCs arise via preformation or epigenesis. Swift (1914) first reported that chicken germ cells arose from the hypoblast. This view was strengthened by Eyal-Giladi *et al*. (1981) who showed that PGCs developed from the epiblast using chick-quail chimeras. Transplantation of blastodermal cells from one embryo to another and *in vitro* culture of blastodiscs showed that PGC precursors were present in the stage X EG&K embryo (Karagenç *et al*. 1996; Naito *et al*. 2001). These reports indicated that avian PGC formation was via epigenesis as opposed to preformation.

Tsunekawa *et al*. (2000) isolated the chicken homolog (*cvh*) to the germ cell specific *Drosophila vasa* gene using primers specific to a putative ATP-binding domain of the mouse *vasa* homolog. Immunohistochemistry using a CVH antibody on pregastrulation chicken embryos showed that *vasa* positive PGC precursors were present in the central region of the area pellucida in the stage X HH embryo. Based on the total number of cells in a stage X blastodisc it was predicted that at this stage there would be approximately 33 PGC precursors present. These *cvh* positive cells co-localized with spectrin proteins (Tsunekawa *et al*. 2000). Spectrin proteins are cytoskeletal proteins that accumulate in the same region as the mitochondrial cloud *in Xenopus* oocytes. It has been suggested that spectrin proteins may have a role in formation of the "germ plasm". The co-localisation of the PGC precursors with the spectrin proteins supports the view that chicken PGCs arise via preformation despite the fact that other maternal factors and a specialized cytoplasmic region have yet to be identified in the early embryo.

1.2 THE EMERGENCE AND MIGRATION OF PRIMORDIAL GERM CELLS IN VERTEBRATES

It has been clearly demonstrated that organisms can be classified in to two groups: those whose germline is specified via preformation and those by epigenesis. In this

next section, PGC development from specification to colonization of the gonad is discussed for several model organisms. This results from the requirement that the PGCs migrate from their site of origin through the developing embryo to the region of mesodermal tissue that will develop into the gonad. Once the PGCs have colonised the gonadal tissue, sex-specific development occurs and the PGCs begin differentiation into the gametes. In this section, the emergence and migration of primordial germ cells will be discussed for zebrafish, mouse and chicken as many of the genetic cues are similar between these organisms and many of the genes involved have similar functions in proliferation, survival and migration of the germ cells.

1.2.1 Zebrafish

Zebrafish are an ideal model to investigate PGC development. In Zebrafish, PGCs arise in four separate regions of the four-cell embryo as a result of germplasm localisation. PGC formation is due to maternal RNAs that localize to an electron dense region situated in the marginal position of the first cleavage plane (Figure 1.2) (Pelegri *et al*. 1999; Knaut *et al*. 2000). Zebrafish homologs for components of the *Drosophila* pole plasm are enriched in this region including, *vasa* and *deadend* (Olsen *et al*. 1997; Yoon *et al*. 1997; Pelegri *et al*. 1999; Köprunner *et al*. 2001; Weidinger *et al*. 2003, Hashimoto *et al*. 2004; Theusch *et al*. 2006). Maternal *vasa* RNA, is a component of the germ plasm whereas *Vasa* protein is present throughout the oocyte at this stage (Knaut *et al*. 2000). The germ plasm segregates into four strips during the first cleavages until each strip occupies one cell of the early embryo. The orientation of the cells is dictated by the first two cleavages and is random with regard to the axes of embryonic development (Abdelilah *et al*. 1994; Raz 2003). For the next four hours of development, the blastomeres of the embryo continue to divide and the germ plasm is retained in the four cells by a mechanism of asymmetrical inheritance (Knaut *et al*. 2000). The embryo then progresses into the sphere stage of development at which point it consists of approximately 4,000 cells. The germ plasm becomes localized to the cytoplasm and is now segregated to the two daughter cells during cellular division (Yoon *et al*. 1997; Braat *et al*. 1999; Weidinger *et al*. 1999; Knaut *et al*. 2000). During this stage the first definitive PGCs emerge and undergo

rapid proliferation to increase cells numbers from four to fifty cells in four small clusters in locations separate from the future gonad.

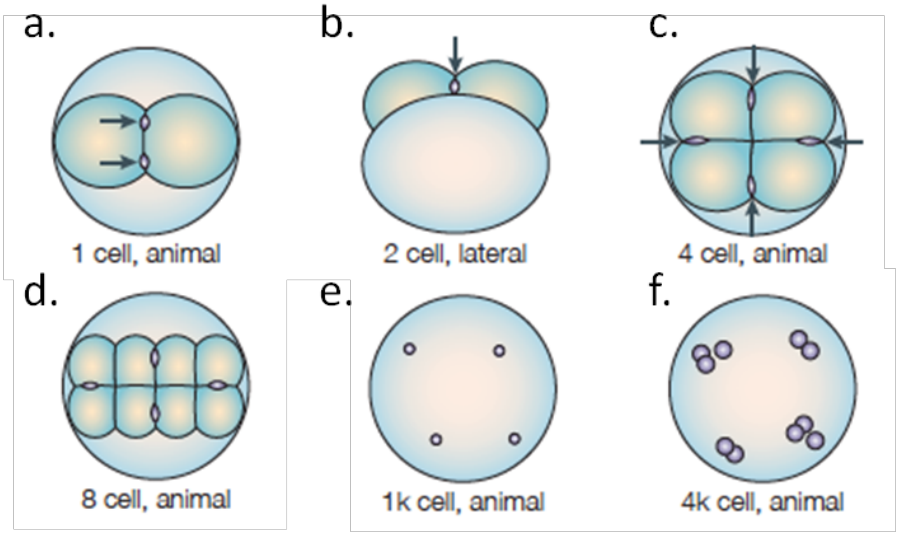


Figure 1.2 Germ cell specification in zebrafish. Schematic drawings of germline development in the zebrafish, during early embryogenesis, based on germ plasm distribution. (a) Germ plasm is first detected at the single cell stage along the first two cleavage planes (black arrows). (b) At the two cell stage germ plasm is located at the animal pole before (c) segregating into four during development to the four cell stage. (d) Asymmetrical segregation of the germ plasm occurs during formation of the 8 cell embryo. (e) By formation of 1K cell there are still only four germ plasm positive cells and by (f) the 4K embryo symetrical segregation of the germ plasm begins. Figure adapted from Raz (2003).

Zebrafish PGCs actively migrate in comparison to other species where PGCs translocate via passive migration (Weidinger *et al*. 2003) (Figure 1.3). The active migration of zebrafish PGCs to the region of the developing gonad was shown to be guided by the chemokine stromal derived factor-1alpha (SDF1a) and its receptor CXCR4b (Doitsidou *et al*. 2002). Using morpholinos to knock-down SDF1a or CXCR4b activity resulted in mismigration of the PGCs, which become scattered throughout the embryo. Prior to migration, PGCs undergo a series of morphological changes to convert them to a highly motile cell (Blaser *et al*. 2005). One germ plasm component essential for the cellular motility is the RNA binging protein Dead end (Weidinger *et al*. 2003). Given that PGCs arise in four separate locations not determined by axes of development the process of migration varies between

embryos. Early embryonic migration consists of six distinctive steps (Weidinger *et al*. 1999). Migration begins at about the 60% epiboly (spread of cell membrane to cover yolk sac) stage when the PGCs become more dorsally located this is followed by the migration of PGCs away from the midline to a more lateral position. By step III of migration the dorsally aligned PGC clusters become positioned along the paraxial mesoderm of the forming embryonic head and body overlying the yolk syncytial layer (YSL) by the 4-somite stage of development. The PGCs then become clustered along the lateral border of the mesoderm. By the 24hpf stage of embryonic development the cells have migrated posteriorly becoming clustered at either side of the body axis parallel to the eighth somite. The PGCs remain in this region where high levels of *SDF1a* can be detected for approximately three hours before migration is resumed (Reichman-Fried *et al*. 2004).

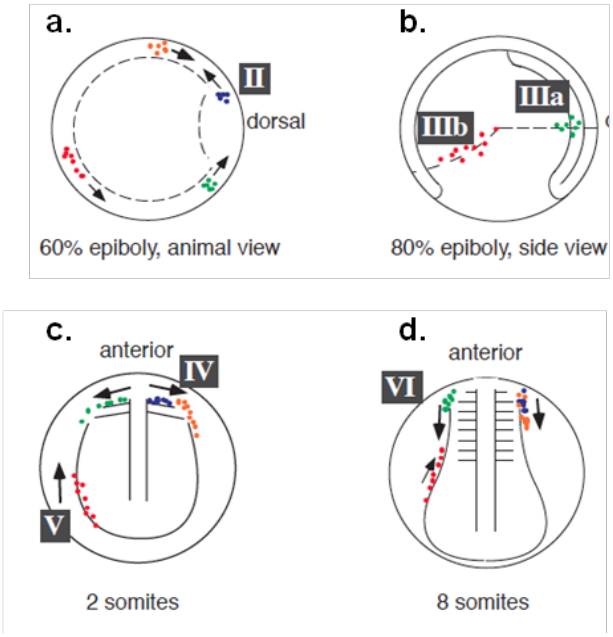


Figure 1.3 PGC migration in zebrafish. Schematic drawings of PGC migration in the zebrafish embryo. (a) 60% epiboly PGCs become more dorsally located (b) Migration of PGCs away from the midline to a more lateral position. (c) By step III of migration dorsally aligned PGC clusters line up along the paraxial mesoderm of the forming embryonic head and trunk. (d) By the 24hpf the cells have become clustered at either side of the body axis parallel to the eighth somite. Figure from Weidinger *et al*. (1999).

Although SDF1a acts as the chemoattractant, spatial information is required for the correct directional migration of the PGCs. This is provided by expression of staufen -1, -2 and HMGCoAR (Ramasamy *et al*. 2006; Thorpe *et al*. 2004; Van Doren *et al*. 1998). It has been shown that PGCs will fail to align along the lateral and anterior borders of the trunk mesoderm when HMGCoAR activity is disrupted resulting in delayed PGC population of the gonads (Thorpe *et al*. 2004). Inhibition of Staufen -1 and -2 activity does not affect formation of PGCs despite resulting in a loss of *vasa* expression. This leads to mismigration of the PGCs and eventual apoptosis (Ramasamy *et al*. 2006). These data suggest that each of the three factors has an individual role in the speed or direction of PGC migration. PGC population of the gonad and subsequent differentiation to form the gametes requires greater investigation to be fully understood.

1.2.2 Mouse

Unlike insects and fish, mammalian PGCs arise through epigenesis, characterised by a lack of germ plasm. As stated previously, alkaline phosphatase-positive cells were indentified in the extra-embryonic mesoderm close to the posterior region of the primitive streak. This has been detailed further by observing the clonal descendants of single epiblast cells injected with a lineage marker at 6.0 – 6.5 dpc. Lineage tracking enabled identification of precursors to the PGCs within the proximal region of the gastrulating embryos next to the extraembryonic ectoderm (Lawson and Hage 1994). When cells from the distal region that normally differentiate into ectoderm were transplanted into the proximal region of stage-matched embryos some of these cells were observed to differentiate into PGCs (Tam and Zhou 1996). This demonstrated that formation of germ cells is not restricted to cells of the proximal region of the epiblast. These findings suggested that site-specific signals might influence cell fate including differentiation to PGCs. These signals were identified when in mice defective for members of the transforming growth factor β (TGFβ) family. These mutant mice were found to be defective for the signalling molecules BMP4 and BMP8b and were either devoid of both PGCs or showed a marked reduction in PGC number (Ying *et al*. 2000 Ying *et al*. 2001; Ying and Zhao 2001).

A third member of the TGFβ family, BMP2, was also shown to be required for mouse PGC formation (Ying and Zhao 2001). BMPs signal through the SMAD intracellular signal transducers. Activated SMAD proteins have been shown to be present in this proximal region of the embryo at 6.0 dpc. This suggests that PGC specification is mediated by BMP4 and 8b expression from the ectodermal cells and BMP2 from endodermal cells (Lawson *et al*. 1999; Ying *et al*. 2000; Ying *et al*. 2001; Ying and Zhao 2001; Matsui and Okamura 2005). Another protein identified as having an essential function in the specification of PGCs is the cell adhesion transmembrane protein E-cadherin. When expression of E-cadherin is blocked in the proximal epiblast at 6.75-7.0 dpc, specification of the PGCs is prevented (Okamura *et al*. 2003).

Clonal analysis demonstrated that by 7.2dpc approximately 45 progenitor cells had differentiated into PGCs and were now lineage restricted (Lawson and Hage 1994). The location of these differentiated cells precisely corresponded to that of the alkaline phosphatase stained cells indentified at 7.25dpc in the extraembryonic mesoderm posterior to the end of the primitive streak (Ginsburg *et al*. 1990). After these early stages of mouse PGC specification, the cells migrate to the region of gonadal development (Figure 1.4). Migration begins around 8.5dpc and is completed by 10-11dpc. PGCs identified in the stages immediately prior to migration express, *B lymphocyte-induced maturation protein-1* (*Blimp-1*), *fragilis* and *stella*. PGC migration begins with the fragmentation of the PGC cluster and coincides with a downregulation of *fragilis*. Fragmentation of the PGC cluster is an essential step in the process for PGC migration. In *blimp1* mutant mice embryos PGCs fail to migrate resulting in a reduction in the number of PGCs in the gonad in heterozygotes or complete absence in homozygotes (Ohinata *et al*. 2005). PGCs that do form in Blimp1-defiecient mutant mouse embryos are reduced in number and form tight clusters of cells that are unable to migrate and proliferate as normal (Ohinata *et al*. 2005). At 8.5dpc the hindgut forms as the endoderm folds inwards, the PGCs are then found along the entire length of the ventral wall in the fully formed hindgut. From this position the PGCs migrate dorsally through the body wall towards the

notochord and the dorsal aorta. The cells then migrate on either side of the embryo to the two genital ridges that have begun to form (Molyneaux *et al*. 2001; McLaren 2003). Migration of the germ cells is mediated by a number of signalling factors and pathways in particular the c-kit/Steel Factor signal transduction pathway. Both c-kit (*White spotting, W*) and *Steel* (*Sl*) mutant embryos display problems with PGC migration and proliferation. In these mutants, specification of PGCs occurs as normal but after 8.5 dpc they fail to migrate or proliferate, instead forming clumps of cells in the floor of hind gut and in some cases in ectopic regions (Mintz and Russell 1957; Buehr *et al*. 1993; Donovan 1994;). It has also been reported that c-kit/Steel factor directly affects PGC survival as observed through germ cells counts at various stages in embryonic development (Dolci *et al*. 1991; Pesce *et al*. 1993). What guides PGC migration in mice has not been fully elucidated although research has shown that like in zebrafish, SDF1 and its receptor CXCR4 do play a role in this process. The SDF1 receptor, CXCR4, is expressed by migratory PGCs whilst *Sdf* RNA is expressed in high levels in the developing gonad and mesonephros at 10.5dpc (McGrath *et al*. 1999; Molyneaux 2003). In CXCR4 and SDF1 mutant embryos PGCs were able to migrate out of the hindgut, however the number of PGCs colonizing the genital ridge was reduced. (Molyneaux 2003; Ara *et al*. 2003). In CXCR4 mutant embryos it was shown that PGCs were able to migrate out of the hindgut but the number of PGCs colonizing the genital ridge was reduced. The reduction of PGCs shows that although not essential for the initiation of migration SDF1/CXCR4 interaction is essential for colonisation of the gonad. All these findings implicate a role for SDF1/CXCR4 in the proliferation and/or survival of PGCs.

Mouse PGCs enter the genital ridge between 10 and 11 dpc and by 12.5 dpc have undergone a variety of transcriptional changes. Transcriptional changes coincide in loss of motility and the beginning of sex chord formation (Molyneaux *et al*. 2001; Molyneaux *et al*. 2004). On entering the genital ridge, the cells begin to express a different set of germ cell-specific markers and are known as gonocytes (McLaren 2003). At this stage, the expression of *TNAP* and *SSEA1* genes is down regulated and for the first time the cells begin to express mouse *vasa* homolog (*mvh*). By 12.5 dpc

the germ cells in both male and female embryos will have undergone at least two more rounds of mitosis and meiotic genes have began to be upregulated including *scp3* (Di Carlo *et al*. 2000). The germ cells in the male genital ridge then undergo mitotic arrest and remain as G0/G1 spermatagonia until mitosis is resumed after birth. Contrastingly, in females the germ cells do not arrest until about the time of birth in the diplotene stage of meiosis (Upadhyay and Zamboni 1982; McLaren 1995).

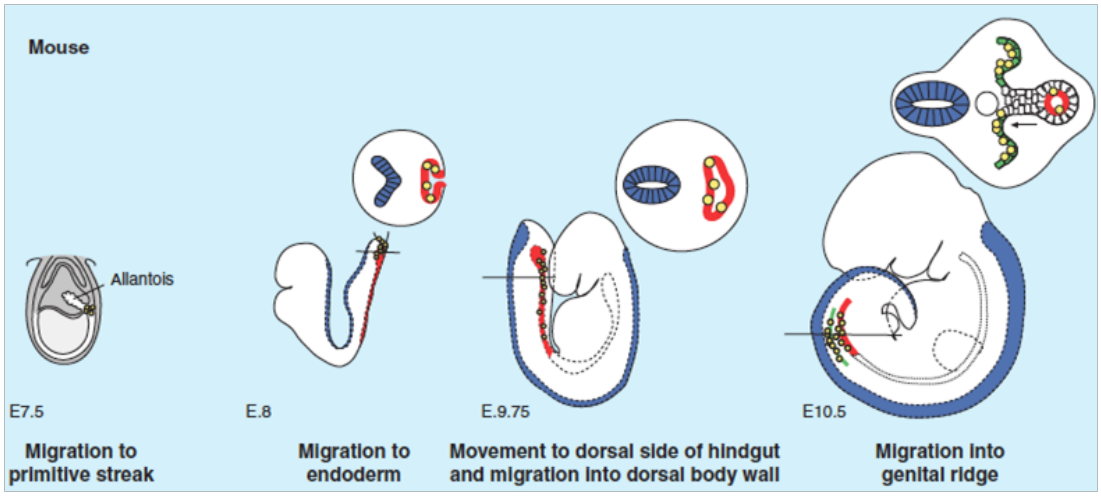


Figure 1.4 PGC migration in the mouse embryo. Lateral view and cross-section of PGC migration in the developing mouse embryo. At E7.5 germ cells migrate towards the primitive streak and then spread along the forming endoderm during E8. At E9.75 the germ cells migrate to the dorsal side of the hindgut into the lateral body wall. By E10.5 most germ cells have colonised the genital ridge. Figure from Weidinger *et al*. (1999).

1.2.3 Chicken

The mechanisms surrounding the emergence and migration of chicken PGCs have not been fully characterised but some of what is known or hypothesised has similarities with what has been described previously in zebrafish and mouse (1.2.1 and 1.2.2). In this section the characterisation, emergence and migration of PGCs in the chicken will be covered.

1.2.3.1. Structure

The general characteristics of PGCs from several chordates, covering all vertebrates and some closely related invertebrates have been outlined in several publications and summarised by Nieuwkoop and Sutasurya (1979). Chicken PGCs were first characterised by Swift (1914) where it was described that an increase in the volume of the nucleus and cytoplasm makes the PGCs in the chicken much larger than the surrounding somatic cells. Chicken PGCs are always round or oval and approximately 15 to 20 microns in diameter with a pronounced, membrane bound nucleus and periodic acid Schiff (PAS)-positive glycogen (Fujimoto *et al*. 1976; Tang and Zhang 2007). The nucleus, 8 to 12 microns in diameter has a pronounced membrane and is usually positioned eccentrically. *In vitro,* chicken PGCs retain their large rounded morphology, granulated cytoplasm, undergo symmetrical division and are non-adherent.

1.2.3.2. Origin

Experimental evidence suggests that chicken PGCs arise via preformation. The chicken oocyte is thought to contain germplasm determinants that specify PGCs although the earliest stage of development at which determinants have been identified is stage X EG&K. At this stage in development, the embryo consists of blastodisc, a single layer of approximately 60,000 cells. Two experiments have identified that germ cell determinants in the blastodisc are located to the central region. Karagenç *et al*. (1996) dissected the blastodisc into two separate regions, peripheral and central, and cultured the sections *in vitro*. They reported that PGCs primarily develop from the central region of the blastodisc. This was confirmed when the central region was dissected out and the manipulated embryos allowed to develop. This dissection resulted in a significant reduction in the number of PGCs observed. The cells in the blastodisc that give rise to the germ cells are known as PGC precursors (pPGC).

Chicken embryonic development from a single layer of cells into the blastula happens between stages XI and XIV (EG&K). The blastula consists of upper epiblast

and lower hypoblast layers surrounding the small cavity called the blastocoel. Karagenç and Petitte (2000) used morphology and staining for stage-specific embryonic antigen-1 (SSEA-1) to identify PGCs isolated from the blastodermal cells of a stage X EG&K embryo. Germ cell determinant *vasa*, was used to identify germ cells in the early stage embryo (Tsunekawa *et al*. 2000). The antibody against the chicken vasa gene homolog (CVH) was used to identify approximately thirty three *cvh* positive cells in the epiblast of stage X EG&K embryos (Figure 1.5a). As the embryo develops the pPGCs increase in number and migrate from the epiblast to the hypoblast where around seventy pPGCs can be identified (Eyal-Giladi*et al*. 1981; Ginsburg and Eyal-Giladi, 1986; Karagenç *et al*. 1996). The embryo then continues to develop resulting in the anterior displacement of the hypoblast and the pPGCs to a region called the germinal crescent at which point they are identifiable as true PGCs (Figure 1.5b) (Ginsburg and Eyal-Giladi 1986). The germinal crescent lies anterior to the region of head development in stage 8 HH (Hamburger and Hamilton 1951) developing embryos. Confirmation of the presence of germ cells within the germinal crescent was first shown in a transplantation experiment. By injecting cell suspension from the germinal crescent of a healthy chicken embryo in to a UV sterilized embryo a normal PGC population was re-established (Reynaud *et al*. 1969).

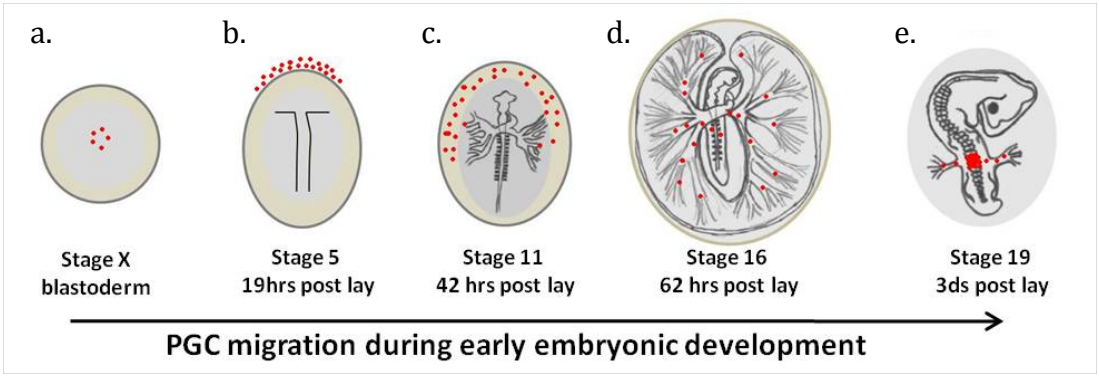


Figure 1.5 PGC migration in the chicken embryo. (a) *Vasa* positive cells are first identifiable in the stage X embryo. (b) Accumulation of PGCs in the anterior germinal crescent. (c) Infiltration of PGCs into the blood islands and the start of transfer into the blood circulation. (d) Circulation of PGCs through the extra- and intra-embryonic vasculature and the beginning of their regress in the region of gonad formation. (e) Continued colonisation of the forming gonad by actively migrating PGCs. Figure adapted from Nieuwkoop and Sutasurya (1979).

1.2.3.3. Migration

Migrating PGCs in the chicken embryo use the vascular system to move from germinal crescent to the developing gonad (Nieuwkoop and Sutasurya 1979; Kuwana 1993) (Figure 1.5c). This begins around stage 11 HH as the blood vascular system begins to form (Nakamura *et al*. 2007). From the circulating blood the PGCs actively migrate out of the vessels via capillaries located close to the germinal epithelium. As has been demonstrated in zebrafish, it is thought *deadend* expression is required for motility of the chicken PGCs and that migration is mediated by an attraction to the chemokine SDF1α (Weidinger *et al*. 2003; Stebler *et al*. 2004). The PGCs migrate on either side of the embryo body from the lateral plate mesoderm along the dorsal mesentery to the developing gonad (Fujimoto *et al*. 1976; Nakamura *et al*. 1988; Kuwana 1993). This process occurs during developmental stages 12 to 17HH. The developing gonad buds off the surface of the mesonephroi (embryonic kidney) and at about embryonic day (E) 4.5 the PGCs become incorporated into the developing gonad. At this stage PGCs are equally distributed between the gonads but as development progresses a preference towards the left gonad can be observed. This is perhaps due to the degeneration of the right gonad in female embryo as the left develops into a functional ovary. PGCs then differentiate in to oogonia in females by E8 (Swift 1915) and spermatagonia in males after E13 (Swift 1916).

1.3 GROWTH FACTORS AND CYTOKINES IMPLICATED IN GERM CELL SURVIVAL

The *in vivo* and *in vitro* research carried out in several organisms including *Drosophila*, zebrafish, mice and chicken has identified key growth factors in the survival and proliferation of PGCs. Extacellular growth factors that are essential to PGC survival propagation and migration, include; stem cell factor (SCF), fibroblast growth factor (FGF), Leukaemia Inhibitory Factor (LIF), IGF1 and SDF1 (Table 1.1)(Dolci *et al*. 1991, 1993; Godin *et al*. 1991; Matsui *et al*. 1991, 1992; Resnick *et al*. 1992, 1998; Pesce, *et al*. 1993, Karagenç and Petitte, 2000, Schlueter *et al*. 2007).

Factor	Chicken	Other vertebrates
FGF2	Proliferation/Propagation	Proliferation
LIF	Propagation	Survival/Propagation
SCF	Survival/Propagation/Migration	Proliferation and survival
SDF-1	Migration (H)	Survival/Migration
IGF1	Poliferation	Survival/Proliferation

Table 1.1 Growth factors and their function. A list of growth factors and their likely function in chicken and mouse PGC survival, propagation, proliferation and migration based on published literature. H = hypothesised.

1.3.1 Stem cell factor (SCF) and signalling pathway activation

Stem cell factor is the ligand for the proto-oncogene c-kit, which encodes a membrane-bound tyrosine kinase receptor. SCF is present in many tissues in two forms, soluble and membrane bound both of which are thought to function differently. SCF/c-Kit interaction activates the PI3K/AKT signalling pathway (Figure 1.6) (Sette *et al*. 2000; De Felici 2000; Liu *et al*. 2007). PI3K/AKT signalling is involved in proliferation, survival, migration, and metabolism. There are three forms of AKT; AKT1, AKT2 and AKT3 each of which has been shown to have a different function. AKT1 knock-out mice although viable are smaller in size and showed shortened lifespans when exposed to γ-irradiation compared to their wild type counter parts (Chen *et al*. 2001). Humans and mice deficient for AKT2 are insulin resistant and develop diabetes or diabetes-like symptoms (Cho *et al*. 2001; George *et al*. 2004). In contrast AKT3 knockout mice show no growth retardation or problems in glucose metabolism but brain development is affected resulting in small brain size in adult mice (Tschopp *et al*. 2005).

SCF stimulates the PI3K/AKT pathway by binding the transmembrane tyrosine kinase receptor, c-kit, which forms a heterodimer and in turn recruits phosphoinositide 3-kinase (PI3K). PI3K then becomes activated by phosphorylation which triggers receptor bound PIP2 to generate secondary messenger PIP3, triggering conformational change and relocation of the AKT molecule to the plasma membrane. At the plasma membrane AKT becomes phosphorylated by PDK1/2. Phosphorylated AKT promotes cell survival by inhibiting the phosphorylation of

apoptotic factors GSK3, BAD, FKHR and Caspase 9 (Stoica *et al*. 2003). AKT molecules are not exclusively activated by an SCF/c-kit mediated response.

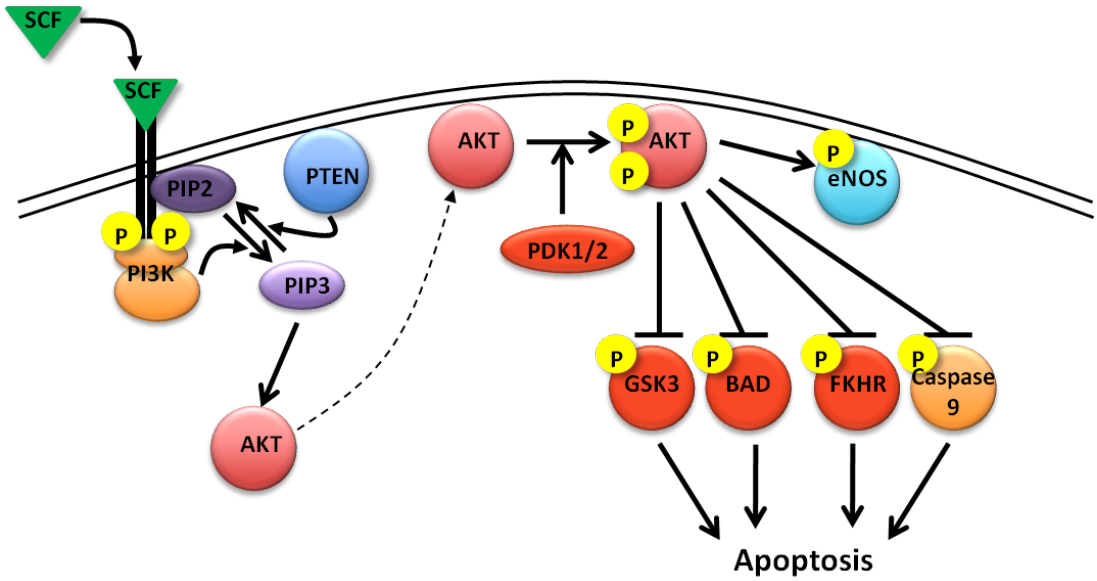


Figure 1.6 PI3K/AKT signalling pathway. SCF binds the transmembrane tyrosine kinase receptor, c-kit, which in turn recruits phosphoinositide 3-kinase (PI3K). PI3K then becomes activated by phosphorylation which triggers receptor bound PIP2 to generate secondary messenger PIP3, triggering a conformational change and relocation of the AKT molecule to the plasma membrane, where it is activated through phosphorylation by PDK1/2. Activated AKT can then inhibit cellular apoptosis.

1.3.1.1 SCF and the PI3K/AKT pathway in mouse PGCs

In the mouse, the c-kit receptor is encoded at the *white spotting* locus (*W*) and its ligand, SCF, is encoded at the *steel* (*Sl*) locus. Mutations in either of these genes results in decrease proliferation of PGCs, failure to migrate properly and increased apoptosis, and in some cases complete sterility has been observed (Mintz and Russell, 1957; Manova *et al*. 1992). Whilst SCF expression has been identified in the somatic cells surrounding and through which the PGCs migrate, c-kit is also expressed by the PGCs and in the genital ridge but not in the stromal tissues (Orr-Urtreger *et al*. 1990). SCF has been shown to be essential for the *in vitro* culture of mouse PGCs by promoting proliferation and survival and inhibiting apoptosis (Dolci *et al*. 1991; Godin *et al*. 1991; Matsui *et al*. 1991; Manova *et al*. 1992; Pesce *et al*. 1993).

Activation of AKT by SCF or other factors is essential for mouse PGCs *in vivo*. Increased PGC proliferation was observed when retroviral-mediated gene transfer was used to induce over-expression of a wild type form of AKT kinase (Sette *et al*. 2000; De Felici 2000; Liu *et al*. 2007). In contrast a decrease in PGC numbers was observed when the dominant-negative form was expressed. This result illustrates the importance of AKT kinase on PGC proliferation *in vivo.* PI3K/AKT signalling is also important in the derivation of mouse EG cells from PGC cultures *in vitro* (Kimura *et al*. 2008). When PI3K/AKT signalling was blocked using pharmacological inhibitors to PI3K no effect on the *in vitro* culture of mouse PGC was observed (De Miguel *et al*. 2002). However, the PGCs referred to in this paper may in fact be EG cells based on the morphology of the cells in the figures.

1.3.1.2 SCF and the PI3K/AKT pathway in chicken PGCs

In contrast to what has been observed in the mouse, little is known about the role of SCF/c-Kit interaction and the PI3K/AKT pathway in chicken PGC survival. There is currently no documented evidence that SCF induced signalling or the PI3K/AKT pathway is necessary for chicken PGC, proliferation, migration and survival *in vivo*. The work carried out on *in vitro* cultures is contradictory. When chicken blastodermal cells were cultured in the presence of chicken SCF, expressed by *E.coli* and purified (Bartunek *et al*. 1996), an increase in the number of PGCs was observed (Karagenç and Petitte, 2000). Van de Lavoir *et al*. (2006) recommended the addition of mouse SCF in culture medium for the propagation of chicken PGCs in culture and reported that removal of SCF may influence the dedifferentiation of chicken PGCs to EG cells. However this was in conjunction with the removal of both the chicken serum and the additional hFGF2 from the medium as well. Recently it was shown that chicken PGCs could be propagated in culture with or without additional SCF (Choi *et al*. 2010). These results suggest that SCF/c-kit mediated signalling may not be required for the *in vitro* propagation of chicken PGCs. The role of PI3K/AKT signalling in chicken PGC survival has not been documented and is investigated in chapter 4.

1.3.2. Fibroblast growth factors and MEK/ERK signalling

Fibroblast growth factors (FGFs) induce signalling by binding FGFRs, receptor tyrosine kinases (RTKs). FGF/FGFR interaction can induce a number of downstream pathways including MEK/ERK (Figure 1.7). MEK/ERK signalling is stimulated when FGFRs bind to extracellular signals resulting in autophosphorylation of tyrosine residues on the receptors intracellular binding domain. Mitogen activated protein kinases (MAPKs) connect the cell surface receptors to their downstream effectors via a cascade of phosphorylation. Two MAPKs that have a role in self-renewal are extracellular signal-regulated protein kinase-1 and -2 (ERK1/2). When the RTK becomes bound by extracellular cues, including FGFs the receptor becomes activated at which point Grb2 adapter protein becomes bound to the RTK at the SH2 domain-phosphotyrosine. Grb2 then interacts with SOS (Son of sevenless), which becomes localised at the plasma membrane. Membrane localized SOS stimulates the conversion of GDP to GTP on Ras, a small G-protein. Activated Ras induces Raf, which then induces phosphorylation of MEK1/2, initiating the MAPK cascade that results in ERK1/2 activation through tyrosine phosphorylation. Activated ERK1/2 can activate cytoplasmic targets and various transcription factors (reviewed by Katz *et al*. 2007). FGF/FGFR interaction can also stimulated the PI3K/AKT pathway. Activation of the MEK/ERK and PI3K/AKT pathways by FGFR mediated signalling is essential during early vertebrate development (Böttcher and Niehrs 2005).

1.3.2.1 FGF2 and the ERK1/2 pathway in mouse PGCs

The isolation of mouse ES cells, EG cells and EpiStem cells have all required FGF2 induced signalling. Expression of the FGF2 has been detected in the mouse gonad and the expression of the FGF receptors 1 and 2 in RNA purified from mouse PGCs (Resnick *et al*. 1998). Addition of FGF2 to mouse PGCs *in vitro* resulted in increased cellular proliferation (Buehr 1997). In mouse PGC cultures the effects were relatively small and required the presence of SCF and LIF. Increased levels of FGF2 in mouse PGC cultures for more than five days resulted in dedifferentiation of the cells to form EG cell colonies (Resnick *et al*. 1992). This effect was confirmed and extended when it was demonstrated that PGCs dedifferentiate into EG cells as a

consequence of adding FGF2 to the culture medium (Durcova-Hills *et al*. 2006). De Miguel *et al*. (2002) showed that inhibition of the MEK/ERK pathway significantly

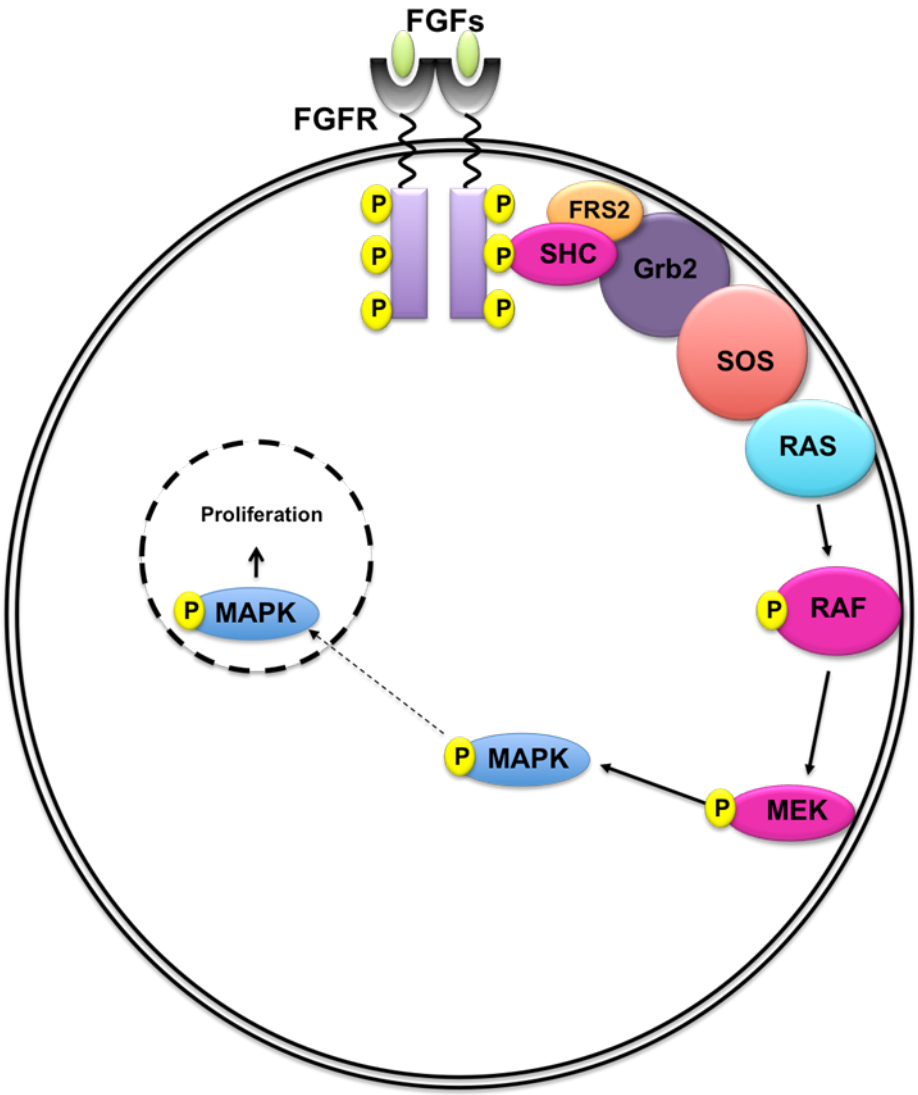


Figure 1.7 MEK/ERK signalling pathway. FGF-2 binds to its receptor, tyrosine autophosphoylation is activated. FRS2 and SHC bind to the phosphorylated tryosine residues providing a dock for the GRB-2-SOX complex. Binding if the complex to SHC activates RAS which I turn activates RAF. Activated RAF induces phosphorylation of MEK which then activates ERK1/2 (MAPK) in the cytoplasm. Phosphorylated ERK1/2 (MAPK) translocates to the nucleus where it promotes proliferation through control of transcription factors.

inhibited mouse PGC growth *in vitro.* FGF2 can also stimulate the PI3K/AKT pathway. Mouse PGCs cultured have been shown to undergo dedifferentiation to EG cells when AKT is hyperphosphorylated as a result of culturing the cells in medium supplemented with FGF2 and LIF (Kimura *et al*. 2007). It has also been reported that germ cells in FGF2-knockout animals undergo normal gametogenesis and that these animals have fertility rates comparable to wild type (Ortega *et al*. 1998). These data suggest that the presence of FGF2 has more important role in PGC survival *in vitro* than *in vivo*. It may also indicate that there are compensative signalling pathways present in the embryo.

1.3.2.2 FGF2 and the ERK1/2 pathway in chicken PGCs

When chicken PGC numbers were observed in blastodermal cells isolated from the stage X embryo EG&K in the presence of bovine FGF2 no increase in PGC number was observed (Karagenç and Petitte, 2000). Despite this result van de Lavoir *et al*. (2006) report the requirement of hFGF2 addition to culture medium for the isolation and propagation of chicken PGCs *in vitro*. Choi *et al*. (2010) have also shown that the presence of hFGF2 in PGC cultures promotes cellular proliferation mediated by activation of the MEK/ERK pathway.

1.3.3 Leukaemia inhibitory factor (LIF) and signalling pathway activation

Leukaemia inhibitory factor is a member of the IL6 cytokine family. LIF signals through the transmembrane receptor homeo- or hetero- dimers of gp130 or gp130 and related receptors including the LIF receptor (LIFR) (Boulton *et al*. 1994; Yoshida *et al*. 1994). Two main pathways ERK1/2 and JAK/STAT can be activated by LIF induced receptor dimerization. Once receptor dimerization has occurred the gp130 recruits JAK, that phosphorylates the tyrosine kinase residues on the intracellular domain of the receptor, creating binding sites for STAT3 (JAK/STAT pathway) (Figure 1.8) or Shp2 (MEK/ERK pathway) (Figure 1.9). In the case of the JAK/STAT pathway, STAT3 becomes receptor bound. Once bound to the receptor STAT3 becomes phosphorylated at tyrosine residue 705 by the JAK molecules. The

phosphorylated STAT3 proteins then become detached from the receptor and are able to form dimers in the cytoplasm. STAT3 dimers become phosphorylated at

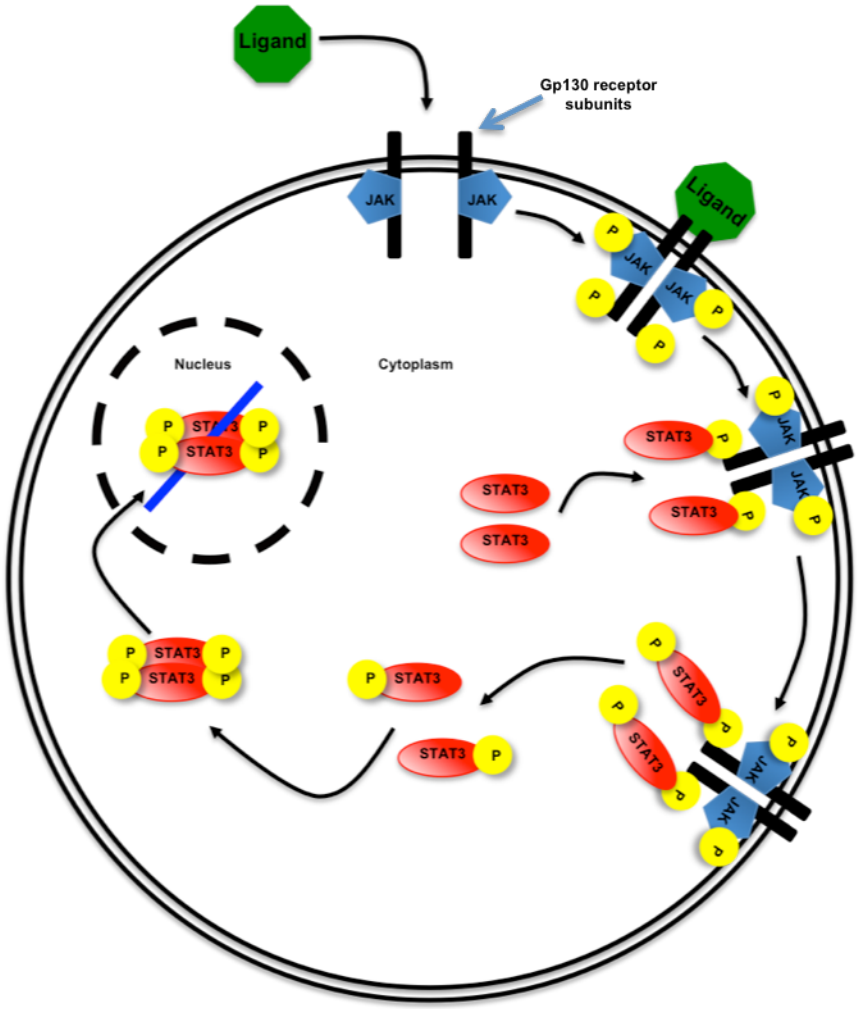


Figure 1.8 JAK/STAT pathway. Binding of the GP130 receptor by one of its ligands induces dimerisation of the transmembrane receptor and phosphorylation of the receptor associated protein JAK. Phosphorylation of JAK induces phosphorylation of the gp130 receptor. The phosphorylated receptor recruits STAT3, which becomes phosphorylated at tyrosine 705 and dissociates from the receptor. Phosphorylated STAT3 proteins dimerize in the cytoplasm, at which point serine 727 becomes phosphorylated and the dimer translocates to the cell nucleus. Within the nucleus STAT3 dimers bind DNA to regulate transcription.

serine residue 727 and translocate to the nucleus where they bind DNA and modulate target gene transcription (Donovan and De Miguel, 2003) . LIF activation of the MEK/ERK pathway is through phosphorylation of the bound Shp2, this in turn activate Grb3 resulting in a signalling cascade as outlined in section 1.3.2 (Burdon *et*

al. 1999). LIF is an important cytokine that promotes survival and self-renewal of PGCs (Farini *et al*. 2005). Receptors for LIF, LIFR and gp130, are present on the cell membrane of mouse PGCs (Koshimizu *et al*. 1996). LIF was identified as an active component in medium conditioned on buffalo rat liver (BRL) cells (Williams *et al*. 1988; Smith, Heath, *et al*. 1988) used previously in the maintenance of ES cell cultures (Smith *et al*. 1988). BRL-conditioned medium is used in the propagation of both mouse and chicken PGCs *in vitro*. The addition of LIF however has been linked to EG cell formation in mouse (Matsui *et al*. 1992), chicken (Park and Han 2000) and rabbit (Kakegawa *et al*. 2008).

1.3.3.1 The role of LIF mediated signalling in mouse PGCs

Despite the link with PGC to EG cell formation, LIF supplementation to *in vitro* mouse PGC cultures has been shown to inhibit the cells from entering meiosis (Chuma and Nakatsuji 2001; Farini, 2005). LIF has not been shown to have the same effect *in vivo*. A LIFR knockout mouse study reported that the morphology and population size of the PGCs in LIFR$^{-/-}$ mice was comparable to the wild type (Ware *et al*. 1995). This was supported in a study using gp130 knockouts (Molyneaux 2003). A slight reduction in PGC number was observed in the testes of male gp130 null mice but not in the females and the mutation had no effect on fertility. LIF might therefore only be important for the *in vitro* propagation of germ cells.

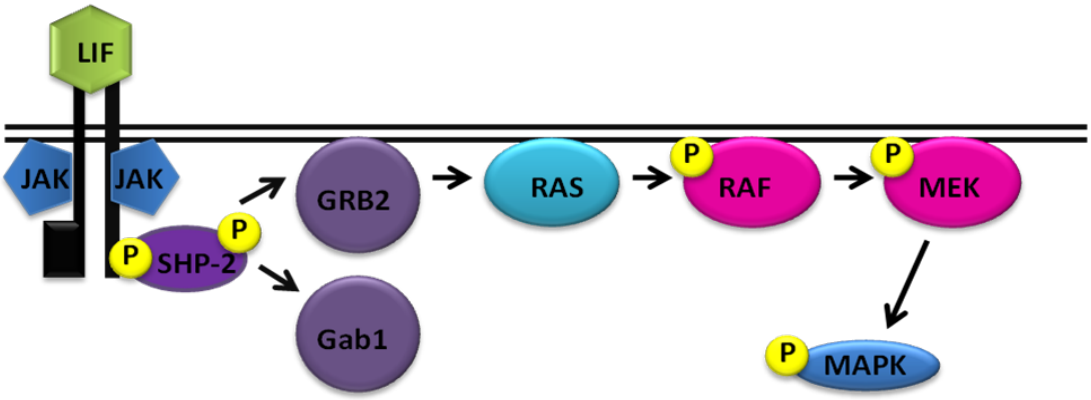


Figure 1.9 LIF/ERK signalling pathway. LIF can induce ERK1/2 (MAPK) phosphrylation via the LIFR/gp130 receptor complex. SHC bind to the phosphorylated tryosine residues providing a dock for the GRB-2 which activates RAS which I turn activates RAF. Activated RAF induces phosphorylation of MEK which then activates MAPK in the cytoplasm. Phosphorylated ERK1/2 (MAPK) translocates to the nucleus where it promotes proliferation through control of transcription factors.

1.3.3.1 The role of LIF mediated signalling in chicken PGCs

BRL-conditioned medium accounts for 50% of the total medium in which chicken PGCs are propagated (van de Lavoir *et al*. 2006). Propagation of PGCs in culture is supported by growth on a layer of mitotically inactivated STO (Sandoz inbred mouse-derived thioguanine- and ouabain- resistant) or BRL fibroblasts (feeder cells). LIF in the culture medium is therefore present in the BRL-conditioned medium and expressed by the feeder cells. FGF2, which is added to chicken PGC culture medium, is known stimulate LIF expression from feeder cells (Rathjen, Toth, *et al*. 1990; Rathjen, Nichols, *et al*. 1990). When LIF is added to culture medium of chicken PGCs grown in the presence of foetal calf serum (FCS) and a chicken embryonic fibroblast feeder layer it has been reported that PGC proliferation is improved (Tang *et al*. 2007). Choi *et al*. (2010) showed that in combination with added FGF2, LIF aided propagation of chicken PGCs in a feeder free environment; however their data appears to show that propagation of the cells was significantly better in FGF2 only conditions compared to FGF2 plus LIF. The requirement for LIF in the propagation and proliferation of chicken PGCs *in vitro* remains unclear. All the documented effects attributed to the presence of LIF are dependent of the presence of other factors.

1.3.4 Insulin-like growth factor (IGF)

IGF signalling pathway is essential for growth and development in vertebrates and is conserved evolutionarily (Molyneaux, 2003). Activation of the IGF pathway is mediated by binding of the IGF-1 receptor (IGF1R) and its ligands IGF-1 or IGF-2 (Molyneaux, 2003). The polypeptide hormone molecule, IGF1, has been shown to be important in embryonic germline development through a series of loss of function studies in zebrafish (Dupont and LeRoith 2001). IGF1R specific morpholino ologonucleotides (MO) were used to knockdown the IGF1 receptor resulting in a marked reduction in the number of PGCs colonizing the gonad (Schlueter *et al*. 2007a; Schlueter *et al*. 2007b). This highlights the role of IGFs in zebrafish PGC migration, specification and proliferation. Addition of IGF1 to chicken PGC culture medium has been indicated to be beneficial to PGC culture (Wang and Du 2004).

This suggests that IGF1 may be important for germline development in the chicken. IGF1 activates signalling through the PI3K/AKT and MEK/ERK pathways (Schlueter *et al*. 2007a; Schlueter *et al*. 2007b).

1.3.5 SDF1/CXCR4 interaction

CXCR4 and CXCR7 are G coupled receptors for the ligand, stromal derived factor 1 (SDF1). SDF1 is a small cytokine also referred to as CXCL12 that has a role in PGC migration and survival in mouse, *Xenopus*, zebrafish and in chicken (Knaut *et al*. 2002; Doitsidou *et al*. 2002; McGrath*et al*. 1999; Molyneaux 2003; Stebler *et al*. 2004; Takeuchi. 2009).

1.3.5.1 SDF1 and PGC survival

SDF1/CXCR4 is involved in germ cell migration and colonisation of the gonads in zebrafish and mouse (1.2.1 and 1.2.2). SDF1/CXCR4 interaction has also been shown to be essential in germ cell survival in mouse, zebrafish and *Xenopus* (Ara *et al*. 2003 Molyneaux *et al*. 2003). Mice deficient in CXCR4 or SDF1 show a reduction in PGC colonizing the gonad compared to wild type and heterozygotes, this has been attributed to a reduction in the survival of the germ cells (Ara *et al*. 2003; Molyneaux *et al*. 2003). It was also shown that addition of SDF1 to tissue slices from the E10.5 embryo resulted in a significant ($P<0.03$) increase in PGC numbers compared to controls in which PGC number was observed to decline (Molyneaux *et al*. 2003). Morpholino knockdown of CXCR4 in *Xenopus* results in a dose dependent decrease in PGC number in the genital ridge. This has been attributed to both survival and migrational defects (Takeuchi *et al*. 2009). Most of the work on SDF1/CXCR4 interaction has been carried out in the zebrafish. Zebrafish have two CXCR4 receptors a and b (Chong *et al*. 2001). Whilst CXCR4a appears to have no function with regard to PGC migration or survival CXCR4b has been shown to be required for migration but not survival (Doitsidou *et al*. 2002). If SDF1/CXCR4 interaction has an effect on PGC survival in zebrafish it is yet to be shown. Both SDF1 and CXCR4 are expressed in the chicken embryo during germ cell migration in regions where germ cells are located such as the germinal crescent

(Stebler *et al*. 2004). It was shown that PGCs migrate to regions of SDF1 expression but the effect on survival has not been assessed (Stebler *et al*. 2004). Given the role SDF1/CXCR4 interaction plays in the survival of PGCs in mouse and *Xenopus,* SDF1 and CXCR4 may be needed for the propagation of chicken PGCs.

1.3.5.2 SDF1 and CXCR7

The SDF1/CXCR7 interaction has a role in degradation of SDF1 to enhance the mitogen gradient for the migration of PGCs in zebrafish (Mahabaleshwar *et al*. 2008; Naumann *et al*. 2010). Binding of SDF1 to CXCR7 is thought to result in internalization followed by degradation of the cytokine. CXCR7 deficiency in mice results in 70% lethality and surviving mice have enlarged hearts indicating a role of CXCR7 in the cardiovascular development (Gerrits *et al*. 2008). The role of CXCR7 in the chicken is unknown.

1.4 METHODS FOR THE PRODUCTION OF TRANSGENIC BIRDS

The production of transgenic chickens has proven useful in investigating vertebrate development and the production of therapeutic proteins. However the complex nature of the chicken reproductive system makes the production of transgenic chickens difficult and at times inefficient. Several methods for the production of transgenic birds have been reported with varying success and will be discussed here.

1.4.1 Manipulation of the zygote

Transgenic mice and other mammalian species can be produced by pronuclear injection of the zygote. Unfortunately this method cannot be used to produce transgenic chickens due to their complex reproductive system. In chickens the time from ovulation to laying of the egg is approximately 24 hours. When a hen ovulates the oocyte is immediately taken up and fertilized in the infundibulum, it then begins to accumulate albumin as it travels along the magnum towards the shell gland/uterus (Figure 1.10). In the uterus, isthmus that forms the eggshell is laid down and the embryo undergoes rapid development. By the time the egg is laid the embryo has already reached developmental stage X EG&K (Figure 1.11). It is therefore only

possible to access the early zygote by sacrificing the hen. Perry (1988) developed a method for the culture of chicken embryos from the zygote stage to hatch.

Microinjection into the pronuclei of chicken zygotes is impossible, as they are not easily visualised beneath the vitelline membrane. To overcome this issue DNA was instead injected into the cytoplasm of the fertilized ova. Love *et al*. (1994) demonstrated that transgenic birds could be produced using this method by crossing G0 chimeras with stock birds. This showed that not only was this a viable method for the production of transgenic birds but that it could be used to integrate large DNA molecules, up to 12.5kb into the avian genome.

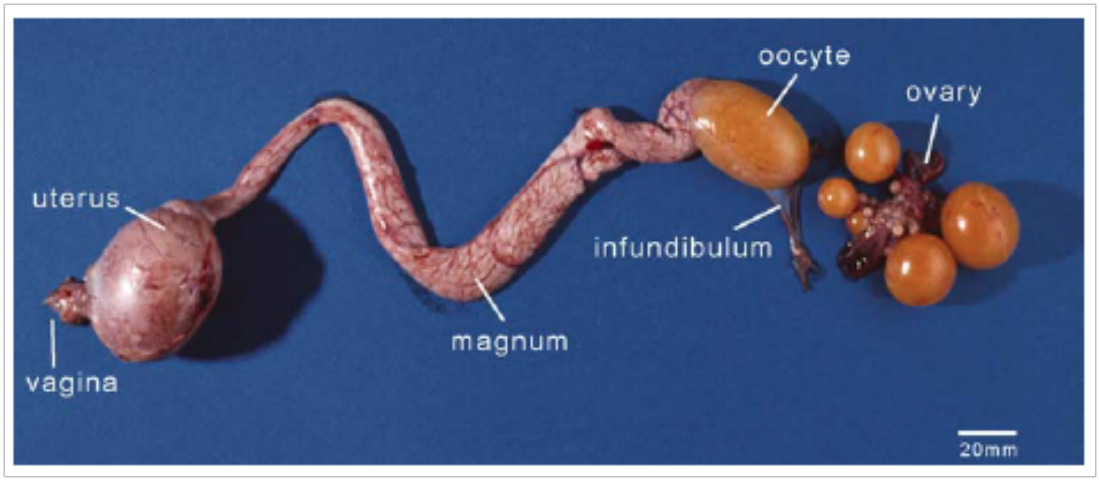


Figure 1.10 Oviduct from a sexually mature adult chicken. The oviduct of a laying hen with developing yolky follicles in the ovary, on the right. A recently released oocyte can be seen infundibulum at the top of the oviduct. As the oocyte passes through the magnum region albumen is synthesised. An egg, just prior to lay is in the uterus or shell gland. By lay the embryo will have developed to form a disc of approximately 60,000 cells. Image taken from Sang (2004).

1.4.2 Chicken ES cells as method for chimera production

The production of transgenic mice using ES cells is extremely efficient and it was assumed that the isolation of chicken ES cells could be equally robust for the production of transgenic birds. A stage X EG&K chicken embryo has two distinct regions (Figure 1.11a), the *area opaca* which gives rise to extra-embryonic tissue and the *area pellucida* which gives rise to the embryo proper. Cells taken from the

area pellucida are pluripotent and have been used to create germline chimeric birds (Petitte *et al*. 1990; Carsience *et al*. 1993; Kagami *et al*. 1995; Kino *et al*. 1997).

1.4.2.1 Blastodermal cell transplantation

By transplanting blastodermal cells from the embryos of Barred Plymouth Rock (BPR) chickens (black feathers) in to stage X EG&K Dwarf White Leg Horn (white feathers) embryos (Petitte *et al*.1990) several somatic chimeras were identified by the presence of both black and white feathers on some of the embryos. One male embryo that survived to hatch was raised to sexual maturity and crossed with BPR hens and produced BPR offspring. This confirmed that the hatchling from the transplantation experiment was also a germline chimera. This method was improved further by the use of γ-irradiation to compromise the cells of the recipient embryo resulting in

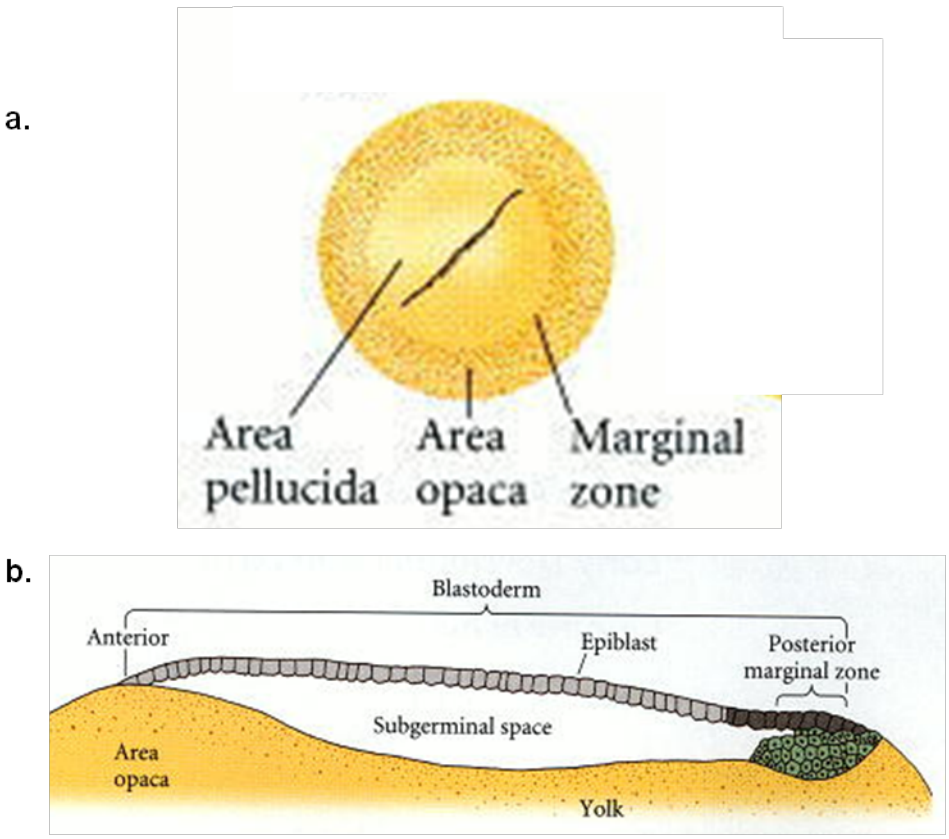


Figure 1.11 Stage X embryo EG&K. (a) Arial view diagram and (b)cross-section of stage X embryo EG&K.

delayed embryonic development. This improved the production of somatic chimeras from 11.3% to 60% and reportedly up to a 100% transmission of donor phenotype to the offspring of the germline chimeras (Carsience *et al*. 1993). Using a *lacZ* reporter construct it was shown that it was possible to transfect blastodermal cells grown in culture for up to seven days (Brazolot *et al*.1991). These cells were injected into recipient stage X embryos and *lacZ* expression in a number of intra- and extra-embryonic regions was identified in stage 11HH embryos. In order to improve the ability to modify the cells a method for culturing them longer than seven days was required.

1.4.2.2 Establishing chicken ES cells

To maintain chicken blastodermal cells in culture for longer periods, Pain *et al*. (1996) using the method for establishing mouse ES cell culture as a basis developed a method for the culture of chicken ES cells. The blastodermal cells isolated from the *area pellucida* (Figure 1.11a) of the embryo were grown on gelatin-coated plates on a layer of mouse STO feeder cells in medium with added FBS, growth factors and cytokines; FGF2, hIGF1, avian-SCF and hLIF, hIL-11. The cells were assumed to be ES cells based on morphology, alkaline phosphatase staining, teleomerase activity and cytokine-dependent proliferation. Some of the first derivations of chicken ES cells although successful did not demonstrate the retention of pluripotency after long-term propagation in culture (Etches *et al*. 1996; Pain *et al*. 1999; Petitte *et al*. 2004). Van de lavoir *et al*. (2006a) showed that chicken ES cells isolated using the Pain *et al*. (1996) method could be propagated in culture for more than 9 months and that even after genetic modification to introduce a GFP reporter construct the cells were still able to contribute widely to the somatic tissues in a host embryo. The production of germline chimeras from chicken ES cells cultured beyond seven days has not been achieved despite the clear demonstration that the blastodermal cells from which the ES cells are established can contribute to the germline. The transmission from the blastodermal transplants is most likely due to presence of primordial germ cell precursors in the transplanted material that can survive only a few days in culture whilst the cells that contribute to the somatic tissues are

maintained. This would explain why after seven days the blastodermal cells/ES cells were no longer able to contribute to the cell line.

1.4.3 Viral vector-mediated gene transfer

Viral vector mediated gene transfer has been used effectively to create transgenic chickens. Salter *et al*. (1987) were the first to report use of viruses as a method for transgenesis. Avian leukosis virus (AVL) was injected into the subgerminal cavity (Figure 1.11b) of stage X EG&K embryos in windowed eggs, the eggs resealed and cultured to hatch. The injected virus infected the blastodermal cells integrating at random into germline progenitor cells. The embryos that survived to hatch were raised to sexual maturity and crossed to produce transgenic offspring carrying viral DNA in all cells. Given the infectious nature of live virus used by Salter *et al*. (1987) replication-deficient viral vectors were developed.

1.4.3.1 The first replication-deficient viral vectors and chicken transgenesis

The first replication-defective retroviral vectors were derived from the mouse reticuloendothelial virus (REV) (Bosselman *et al*. 1989) and from ALV (Salter and Crittenden 1989). Both vectors were successfully used to produce genetically modified birds but at low frequencies, 2-8% using REV and only 0.7% with ALV. These results were further compounded when poor transmission and low transgene expression were observed (Rapp *et al*. 2003). An improvement in the production of germline transgenics but not transmission was observed using a vector derived from the spleen necrosis virus (Mozdziak *et al*. 2003).

1.4.3.2 Lentivirus-derived replication-deficient viral vectors and chicken transgenesis

The development of vectors from the lentiviruses had two significant advantages, they could transfect non-dividing cells (Naldini *et al*. 1996) and were developed from retroviruses, which had been used previously to efficiently produce transgenic animals (Lois *et al*. 2002; Pfeifer *et al*. 2002; Hofmann *et al*. 2003). As well as

producing transgenic pigs and cattle, lentiviral vectors have been used efficiently to produce germline transgenic chickens that express therapeutic proteins in the ovalbumin or block the onward transmission of avian influenza (McGrew *et al*. 2004; Lillico *et al*. 2007; Lyall *et al*. 2010). Although lentivirus has been used successfully to create transgenic chickens, the frequency at which founders are produced and, in turn, transgenic offspring is still relatively low.

1.4.4 Primordial germ cells as tool for producing transgenic birds

Although transgenic birds have been created with some success using retroviral vectors, PGCs offer significant potential for the production of transgenic birds. It was demonstrated that chicken PGCs could be transferred between embryos and still give rise to functional gametes (Reynaud 1976; Wentworth *et al*. 1989). Chicken PGCs isolated from the germinal crescent (stage 7 HH), embryonic blood (stage 17 HH) and the gonads (stage 30 HH) are all capable of forming functional gametes in a host gonad (Han *et al*. 2002; Tajima *et al*. 1993; Vick *et al*. 1993). Germline transmission of donor PGCs can be improved by partial sterilisation of host birds using busulfan toxicity (Vick *et al*. 1993), removal of the central portion of the blastodisc (Kagami *et al*. 1997) or removal of embryonic blood containing circulating PGCs (Naito *et al*. 1994).

1.4.4.1 Retrovirus-mediated modification of chicken primordial germ cells

The first report of chicken PGC modification used the replication-defective AVL retrovirus-derived vector to generate founder birds that transmitted the transgene to their offspring (Vick *et al*. 1993). Donor embryonic blood containing an estimated 3-5 chicken PGCs and virus-derived vector carrying a reporter gene were injected into the vasculature of host embryos. Host embryos that survived to hatch and raised to sexual maturity did transmit the transgene to their offspring. Donor PGC transmission was confirmed by the production of transgenic birds with the donor PGC phenotype. Recently lentiviral vectors were used to transduce chicken PGCs isolated from both the blood and the gonad grown in culture of 2 days (Motono *et al*.

2010). The chicken PGCs were injected in to the blood stream of a host embryo and all males were positive for the transgene and transmitted the transgene at a rate of 3-7%. Although rates of transmission were adequate, the viral vectors that can be used are limited. Many virus-derived vectors require the target cells to be actively dividing. The key to developing chicken PGCs for transgenesis will require a robust culture method.

1.5 *IN VITRO* CULTURE OF GERM CELLS

1.5.1 Mouse primordial germ cell

Mouse PGCs can be isolated from different stages of embryonic development using a variety of sorting procedures and monoclonal antibodies for PGC surface markers (De Felici 2001). Cultured, mouse PGCs can be identified morphologically as large, round floating cells with characteristic blebbing and pseudopodia (De Felici and McLaren, 1983) or by staining for markers of pluripotency (Brinster and Harstad, 1977; Donovan *et al*. 1986; Ginsburg *et al*. 1990; Matsui *et al*. 1992; Durcova-Hills *et al*. 2001). However sustaining PGCs in culture has not been possible beyond just a few days, after which the cells die. The first sustained culture of mouse PGCs *in vitro* beyond just a few days was achieved by addition of FGF2 to the culture conditions (Resnick *et al*. 1992; Matsui *et al*. 1992). This stopped the cells from dying but did cause the PGCs to dedifferentiate to from embryonic germ (EG) cells. As with PGCs, EG cells are positive for SSEA-1 antigen and alkaline phosphatase (ALP) expression (Resnick *et al*. 1992). EG cells have been demonstrated to contribute to somatic tissues and to contribute to the germline (Labosky *et al*. 1994). However, EG cells have been shown to be more similar to ES cells that PGCs in gene profile (Durcova-Hills *et al*. 2008). Analysis of genes that are differentially expressed in PGCs and EG cells showed that EG cells were more similar to ES cells than PGCs. As the propagation of mouse ES cells is well established, this may explain why it has been possible to culture mouse EG cell *in vitro* rather than mouse PGCs.

1.5.2 Chicken primordial germ cells

As observed in mouse PGCs, the culture of chicken PGCs *in vitro* has proved challenging despite the cells having been well described and characterised. Manipulation of chicken PGCs for the purposes of producing transgenic poultry offers a relatively straightforward method for the transmission of induced genetic modification to the next generation.

1.5.2.1 Isolation of chicken PGCs

It has been demonstrated by several laboratories that chicken PGCs isolated from the circulating embryonic blood or the extra-embryonic germinal crescent when injected into host embryos will undergo differentiation and meiosis as normal, forming functional gametes evidenced by the production of offspring with donor PGC phenotype (Wentworth *et al*. 1989; Vick *et al*. 1993; Vick *et al*. 1993; Petitte *et al*. 1991). Although chicken PGCs could be isolated the collection methods proved difficult with cells being collected in small numbers. To increase the number of chicken PGCs that could be collected a Ficoll density gradient centrifugation method was developed to collect chicken PGCs from embryonic blood. Using this method it was possible to inject 100 chicken PGCs into host embryos, this resulted in the successful production of donor-derived offspring with efficiencies ranging from 0 to 11.8% (Tajima *et al*. 1993). This was further improved by the availability of greater starting numbers of chicken PGCs available for transfer by isolating chicken PGCs from the developing gonad (Chang *et al*. 1995; Chang *et al*. 1997; Tajima *et al*. 1998; Park *et al*. 2003). This demonstrated that chicken PGCs could be isolated in large numbers from the developing gonad, form functional gametes evidenced by transmission of donor-derived phenotype, although transmission rates were extremely low.

1.5.2.2 Improving donor chicken PGC germline transmission

Improvements in the germline transmission of chicken PGCs isolated from the gonad were reported when assessing the effects of maintaining chicken PGCs in culture on transmission rates (Park *et al*. 2003). Chicken PGCs were isolated, both with and

without using the Ficoll separation method, from the gonads of stage 28 HH Korean Ogol chicken (KOC) embryos and seeded on to 96-well culture plate. Isolated chicken PGCs were maintained in culture for zero, five and ten days prior to injection in to the host. The conditions used to propagate the chicken PGCs were previously outlined for the culture of chicken EG cells for four months (Park and Han 2000). The cells were cultured in a medium of DMEM supplemented with human SCF (hSCF), murine leukaemia inhibitory factor (mLIF), human interleukin-11 (hIL-11), human insulin growth factor-I (IGF-I), human FGF2 (hFGF2), foetal calf serum (FCS; Gibco), chicken serum (CS; Gibco), L-glutamine, 1mM sodium pyruvate, 1x nucleosides, 1x non-essential amino acids, β-mercaptoethanol, penicillin and streptomycin. Chicken gonadal stroma cells were used as feeder cells, seeded at the same time, as the isolated chicken PGCs. The chicken PGCs survived but proliferated slowly and by day seven in culture began to form adherent colonies on the confluent stroma cell monolayer. Individual stage 17HH White Leghorn chicken (WL) embryos were injected with one hundred and fifty to two hundred KOC PGCs that had been maintained in culture for zero, five or ten days. No effect on chimera production was observed as a result of either the Ficoll treatment or period of time in culture. When the chimeras were assessed for transmission it was found that as the time in culture increased there was a significant increase in germline transmission from 7.8% at zero days to 49.7% in the birds that had been injected with chicken PGCs cultured for ten days. It was observed that Ficoll separation significantly reduced the transmission rates most notably in the ten day culture group where the transmission rate was over 40% in the non-ficoll treated group and between 10 and 25% when Ficoll treatment was used. It was concluded that *in vitro* culture improved germline transmission and suggested that an established system for the culture of chicken PGCs would aid in the development of transgenic birds.

1.5.3 Long term in vitro culture of chicken primordial germ cells

Long term, *in vitro,* culture of chicken PGCs was described by van de Lavoir *et al*. (2006). In this report it was demonstrated that chicken PGCs could be propagated in

culture for several months whilst retaining the ability to form functional gametes. In order to isolate chicken PGCs one to five microlitres of blood was aspirated from the vasculature of Barred Plymouth Rock (BPR) chicken embryos that had been incubated at 37^{o}C until they were between stages 14 and 17 HH of development. The aspirated blood was deposited into wells of either a 46- or 96 well plate that had been seeded prior to aspiration with mitotically inactivated fibroblast cells (feeders). The two cell types used as feeders were buffalo rat liver (BRL) cells and Sandoz inbred mouse-derived thioguanine-resistant and ouabain-resistant (STO) fibroblasts, seeded at 1 x10^{5} and 3 x 10^{4} cells/cm^{2} respectively. The aspirated blood was placed in a complex medium of KO-DMEM, foetal bovine serum (FBS), chicken serum, BRL conditioned medium (Petitte 2004), glutamine, pyruvate, nucleosides, non-essential amino acids, β-mercaptoethanol, SCF and human recombinant FGF. The cultures were maintained for seven to fourteen days during which time the red blood cells apoptose and the chicken PGCs become visible. Using reverse-transcription PCR (RT-PCR) expression of the germ cell-specific genes *CVH* (Tsunekawa *et al*. 2000) and *DAZL* (deleted in azoospermia-like) were identified validating that the cultured cells were chicken PGCs. Further validation was made using FACS profiling to identify CVH and the ovomucin-like protein (OLP), which is expressed on the surface of migratory PGCs (Halfter *et al*. 1996). Chicken ES cells, chicken EG cells and the cultured chicken PGCs were analysed and both CVH and OLP were found to be restricted to the cultured chicken PGCs. The cultured chicken PGCs were also identified to be positive for telomerase activity, using TRAP (telomeric repeat amplification protocol) assay, a distinguishing feature of immortal cell lines. After propagation in culture for at least 35 days several cultured chicken PGC lines were tested for their ability to form functional gametes. Cells from eight male and two female cell lines were injected into White Leghorn (WL) chicken embryos that were between stages 13 and 15 HH of embryonic development. All the male chimeras produced from the male PGC lines and eight of the ten female chimeras from the female PGCs lines transmitted the BPR phenotype to their offspring at transmission rates ranging from less than 1% to 86%. The variability in transmission observed between chimeras produced from the same PGC line was attributed to variability in

the number of cell received by the host embryo at time of injection and difference in cellular proliferation. Mixed sex chimeras, male cells into female host / female cells into male host, did not transmit the donor cell phenotype. This was similar to results observed by Naito *et al*. (1999) where freshly isolated chicken PGCs were injected into host birds. Same sex chimeras showed high level donor phenotype transmission, up to 40% whilst mixed sex chimeras only transmitted donor cell phenotype at rates between 0.1 to 0.9%.

It was noted that within chicken PGC cultures some of the cells would become adherent to the feeder layer. These cells were morphologically similar to ES cells and when FGF, SCF and chicken serum were removed from the culture medium could be expanded and propagated in culture. These cells were determined to be embryonic germ (EG) cells, derived directly from the chicken PGCs by Southern blot analysis of EG cells derived from the GFP expressing transgenic chicken PGCs.

1.5.4 Genetic modification of in vitro culture of chicken primordial germ cells

1.5.4.1 Electroporation with plasmid DNA

Culture PGCs have been genetically modified using plasmid DNA introduced to the cells by the method of electroporation (Van de Lavoir *et al*. 2006). Initial attempts to integrate DNA were unsuccessful and attributed to the effect of gene silencing, which occurs through epigenetic rearrangements of the genome, such as chromatin condensation resulting from DNA methylation. To counter act the effects of gene silencing, insulator sequences (HS4), isolated from the chicken β-globin locus were inserted either side of the GFP (green fluorescent protein) expressing transgene. These sequences have two functions, to block the activity of enhancer elements upstream and to act as a barrier to the silencing effects of chromatin condensation (Figure 1.12) (Burgess-Beuss *et al*. 2002). Insertion of these insulator sequences worked successfully as a barrier to silencing as evidenced by the production of a number of different transgenic male chicken PGC lines some of which were shown to transmit through the germline (van de Lavoir *et al*. 2006). However stable

transfection frequency was low as observed when only one CAG-neo positive clone was identified in the cultured chicken PGCs. This was from a total of 20 transfections using $1x10^{8}$ PGCs per transfection equating to a stable transfection rate of $5x10^{-7}$%. This was far lower that the transfection rate of 0.001% achieved in chicken ES cells using the same method (Leighton *et al*. 2006). However after a total of 135 days in culture these modified chicken PGCs were shown to retain the ability to transit through the germline producing eight potential chimeras, seven of which transmitted the PGC derived genotype at frequencies from 1% to 96%. A total of 46% of the BPR offspring where identified to be positive for expression of the GFP transgene.

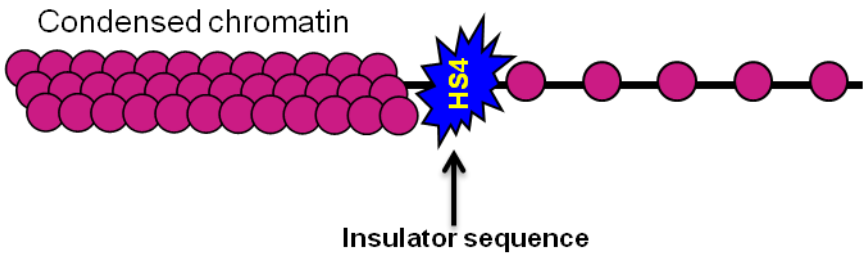


Figure 1.12 Insulator sequence HS4. HS4 insulator sequence can function as a barrier to encroaching chromatin condensation. Adapted from Burgess-Beusse *et al* 2002.

1.5.4.1 Modification of chicken PGCs using ΦC31 integrase

To improve on the very low rate of stable transfection achieved in chicken PGCs using plasmid DNA plus insulators, Leighton *et al*. (2006) utilised the ΦC31 integrase system which had been shown to work effectively in vertebrates including human, mouse, and *Xenopus* (Groth *et al*. 2000; Thyagarajan *et al*. 2001; Olivares *et al*. 2002; Belteki *et al*. 2003; Allen and Weeks 2005). ΦC31 integrase catalyses site-specific integration between attB and attP sites. AttP sitesaere present in the BPR chicken genome. The cultured chicken PGCs were electroporated with a CAG promoter controlled ΦC31 integrase gene plasmid and a GFP reporter construct with an attB site either with or without insulator sequences. A maximum stable transfection rate of 0.001% was achieved in the presence of the insulator sequences and a stable transfection rate of 0.0004% in the constructs where the HS4 was not

present. With insulators, the result was comparable to what had been observed in the chicken ES cells transfected with the plasmid DNA. When the insulator knock-out vector transfection rate was reduced but the efficiency was still a 100-fold higher that those achieved when using the DNA plasmid plus insulator construct. This demonstrated that the ΦC31 integrase system significantly increase transfection efficiency in cultured chicken PGCs. The integration site of transfected PGCs clones were identified by cloning genomic junction fragments. The majority, 25 of 35, insertions were near promoter regions, upstream of the transcription start sites. In the insulator knockout transfections integration was biased towards promoter regions suggesting that integrations that occurred in other areas of the genome were likely to be silenced.

Given the improved rates of transfection observed using the ΦC31 integrase, it indicated that other integration vectors could be useful for the modification of chicken PGCs. Transposons have been utilised in many vertebrate species, embryos and cell lines and offer significant potential as a tool for the trangenesis of cultured chicken PGCs.

1.6 TRANSPOSONS

Prokaryotic genomes contain regions of mobile DNA known as transposable elements or transposons. The first documented observation of transposons was in maize by Barbara McLintock during the 1940s. However it wasn't until data was reported in the 1980s that her discovery of the *Dissociation* and *Activator* (Kempen and Windhofer 2001) elements were confirmed to be transposons. A growing number of transposable elements have been identified in most organisms. Genome studies show that transposable elements contribute to large portions of the genome from 10% in fish species, 37% in mouse, 45% in humans and to more than 80% in some plants such as maize (SanMiguel *et al*. 1996; Lander *et al*.2001; Waterston *et al*. 2002; Muñoz-López and García-Pérez 2010). The definition of a transposable element is a DNA sequence that is able to move from one locus in the genome to another. Many transposons have been identified to share structural and functional

characteristics and can be categorized into two classes, which can then be subdivided into families.

1.6.1 Classes of transposable element

The two classes of transposable element are retrotranposons (Class I) and DNA transposons (Class II). Retrotransposons move via a "copy and paste' method where by the transposable element is replicated to form a copy which integrates in to a different region of the genome whilst the template element remains in its original genomic position (Figure 1.13a). DNA transposons move via a "cut and paste" mechanism involving excision of the transposable element from one locus in the genome and integration into another (Figure 1.13b). Retrotransposons can accumulate in number whilst DNA transposons do not. The focus of this section will be to discuss DNA transposons and their use as gene transfer vectors. There are several superfamilies of transposable elements named after Tc1/Mariner, isolated from *Drosophila* and *C elegans*, piggyBac from frog and *Tol*, which was isolated from the genome of the medaka fish. Within each superfamily there are several transposable elements.

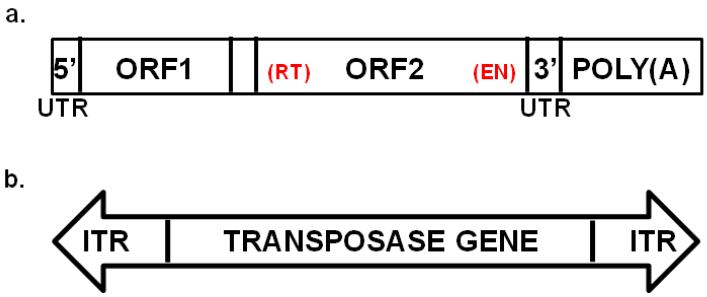


Figure 1.13 Basic structure of two classes of transposable elements. (a) Class I Retrotransposon consisting of a 5'-UTR, internal promoter activity and two open reading frames (ORFS). ORF one encodes a nucleic acid binding site and ORF two encodes protein for replication (RT: reverstrancriptase) and integration (EN: endonuclease). (b) Class II DNA transposon element consisting of a single transposase encoding region flanked by inverted terminal repeats (ITR)

1.6.1.1 Class II transposon mobilisation

DNA transposons consist of a transposase gene flanked by inverted terminal repeats (ITRs) (Figure 1.14a). Transposable elements are mobilized by expression of the

transposase which produces two molecules that recognise and bind to sites on the terminal repeats (Figure 1.14.b). The transposase then cleaves the DNA, releasing the transposable element (Figure 1.14c). The transposase molecules then dimerize resulting in the formation of the *Paired-End Complex* (PEC) (Figure 1.14d). The PEC then binds to a new region of genomic DNA, which is cleaved via nucleophilic attack. Once the DNA has been opened the transposable element becomes integrated (Figure 1.14e).

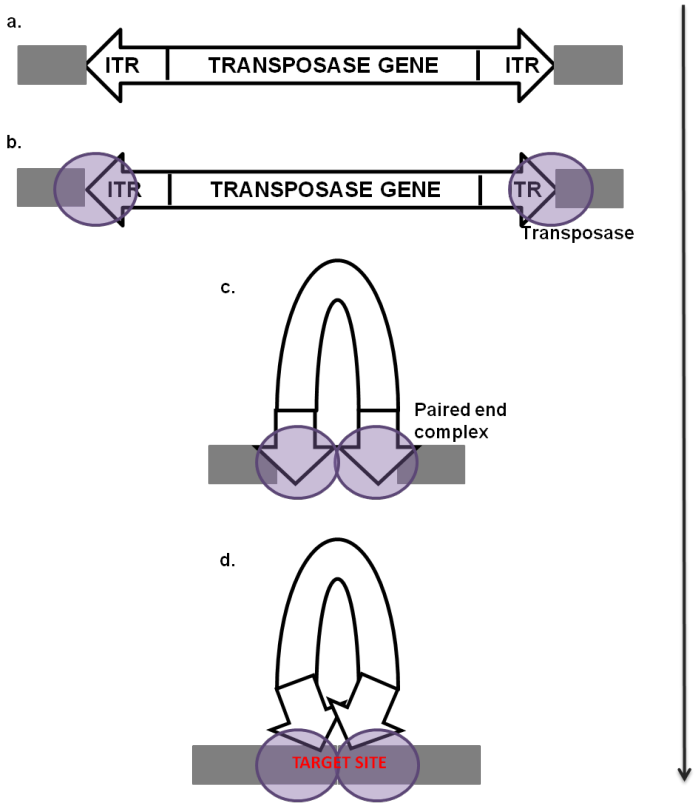


Figure 1.14 "Cut and paste" mechanism of transposition. Diagram outlining the mechanism in which a DNA transposon is mobilised. (a) DNA transposon in genome, (b) expressed transposase molecules bind ITRs, (c) inverted terminal repeats brought together (Paired end complex) and transposon excision takes place (d) excised transposon inserts into target site.

1.6.2 Transposon families

1.6.2.1 Tc1/*mariner* superfamily

The Tc1/*mariner* superfamily is the most widely distributed family of transposable elements having been identified in organisms including *C elegans*, mouse, fungi, plants, insects and fish (Robertson 1993; Plasterk *et al*. 1999). Of the transposons catergorized into this group only ten are known to function naturally, including *minos*, *Mos1* and Tc1 (Emmons *et al*. 1983; Collins *et al*. 1989; Franz and Savakis 1991; Medhora *et al*. 1991; Hartl 2001), with four more having been reconstructed from inactive elements, including *sleeping beauty (SB)* and *frog prince* (Radice *et al*. 1994; Miskey 2003). Like all transposable elements Tc1/*mariner* elements encode a transposase flanked by two ITRs. Despite this the transposase protein encoded by each element differ vastly in sequence whilst retaining two very characteristic domains (Franz and Savakis 1991; Hartl 2001; Medhora *et al*. 1991; Emmons *et al*. 1983; Collins *et al*. 1989). Tc1/Mariner elements target TA dinucleotide regions within regions of the genome where insertion will result in the inhibition of protein formation (Vigdal *et al*. 2002). The *mariner* transposable element isolated from *D.mauritiana* was shown to be effective at integrating into the chicken genome (Sherman *et al*. 1998). By injecting chicken zygotes with a plasmid carrying *Mos1* an active *mariner* element it was possible to produce chimeric birds. 20% of embryos surviving to hatch were positive for at least one copy of the *mariner* element. One cockerel, positive for the transposon in its semen went on to father offspring, 30% of which were identified to have the *mariner* element. This was the first demonstration that transposable elements function in chickens and demonstrated that transposons can be used to modify the chicken genome.

1.6.2.2 *hAT* superfamily

Three transposable elements; *Hobo* from *Drosophila*, *Acitvator* (*Ac*) from maize and *Tam3* from snapdragon are all members of *hAT* (*hobo*, *Ac* and *Tam3*) family of transposons (Calvi *et al*. 1991). These transposons have two characteristics; they generate 8bp target site duplications as a result of the transposition event and they have terminal inverted repeats between 5 and 27bp in length. *hAT* elements range in

size from 4kb on average to as large a 12kb. The following section focuses on the transposable element *Tol2*, a member of the *hAT* transposon family.

1.6.2.2.1 Discovery of Tol2 transposable element

Tol2 was discovered when a recessive mutation causing albinism in the Japanese medaka (*Oryzias latipes*) fish was isolated (Koga *et al*. 1995; Koga *et al*. 1996) and was found to be due to insertion of a novel transposon. *Tol2* is thought to have diverged from a common ancestor with other *hAT* elements, *Ac*, *hobo*, *Tam* and snapdragon (Atkinson *et al*. 1993). A plasmid carrying a *Tol2* element with a mutation in the putative transposase region was injected into zebrafish embryos. The element could no longer be excised from the injected plasmid. Transposition was restored by co-injection of the mutated *Tol2* element and full length *Tol2* mRNA (Koga *et al*. 1996). Transposition of a *Tol2* element identified the presence of a functional transposase within the *Tol2* element. *Tol2* element has no sequence specific preference but shows a propensity to integrate into AT rich regions and transcriptional units (Kondrychyn *et al*. 2009).

1.6.2.3 *piggyBac* superfamily

Originally described as the IFP2 element, *piggyBac* is a transposable element isolated from the genome of the cabbage looper moth, *Trichoplusia ni*. PiggyBac was identified as the result of host response to viral infection in moths infected with nucleopolyhedrovirus (NPVs) (Carey *et al*. 1989). The virus would exhibit FP (Few Polyhedra) plaque mutations due to the insertion of a sequence of host DNA. The structure of the host insertion was a 2.47kb region with 13bp perfect inverted terminal repeats and 19bp subtermimal repeats, flanking a 2.1kb transcriptional unit. The transcriptional unit encodes a single 1.8kb transposase gene with a molecular mass of approximately 64 kDa (Elick *et al*. 1996). Integration of *piggyBac* in the genome is targeted to TTAA sites (Fraser *et al*. 1996; Cadiñanos and Bradley 2007) and a preference for integration into transcription units particularly introns has been reported (Wilson *et al*. 2007). Interestingly, the piggybac transposon elements are able to excise from the genome without leaving a footprint (Elick *et al*. 1996). There

is a high level of conservation between independently isolated piggyBac elements indicating that the transposons DNA sequence is involved in its movement and maintenance (Wang and Fraser, 1993). Fraser *et al*. (1995) showed using baculovirus genome as target that the piggyBac elements could spontaneously integrate into the bacterial genome and cause mutagenesis. Fusion constructs removing a portion of the element sequence and expressing it in *trans* confirmed that mobilization of the piggyBac element was either entirely controlled or at least enhanced by the products it encoded.

1.6.3 DNA transposon-derived vectors

1.6.3.1 Vector design

DNA transposon derived vectors work via a binary system of a "donor" plasmid carrying a gene conferring resistance, fluorescent marker or gene of interest and a second "helper" plasmid carrying the transposase gene. Transposition is controlled by expression of the transposase in trans. These vectors may be functional in a wide range of species making transposons a useful tool for introducing foreign genes into genomic sequence (Figure 1.15).

1.6.3.2 Limitations of a transposon-derived vector capacity

Ding *et al*. (2006) explored the limitations of increasing transposon size on transposition efficiency. It had been previously reported that increases in the length of the Sleeping Beauty transposon correlated directly with a decrease in its transposition events in HeLA cells (Izsvak *et al*. 2004). Using piggyBac vectors containing inserted DNA ranging from 4kb to 14kb to create transgenic mice, it was demonstrated that a reduction in transposition was only observed when inserts exceeded 9kb. It was also reported that transposition, although still possible, was greatly reduced with an insert of 14kb. As a result of this work piggyBac was identified as a useful tool for mammalian transgenesis.

Initial studies carried out using piggyBac have been successful in highlighting its potential in human gene therapy and mammalian genomics. Wu *et al*. (2006) carried

out a series of comparative experiment. They examined the effectiveness of piggyBac in four different mammalian cell lines, three human and one hamster, compared to three other transposons, SB, *Tol2* and *Mos1*. PiggyBac was found to be the most effective transposon system and the only one to be functional in all three cell lines. An increase in the ratio of "helper" plasmid to "donor" plasmid resulted in a rapid decrease in piggyBac ability to transpose, suggesting that piggyBac is inhibited by an overproduction of transposase. Despite this it was suggested that piggyBac may be the most effective transposon for the manipulation of mammalian genomes as it had been demonstrated to be more efficient than SB in transpositional assays. SB had been used preferentially in most previously published mammalian work.

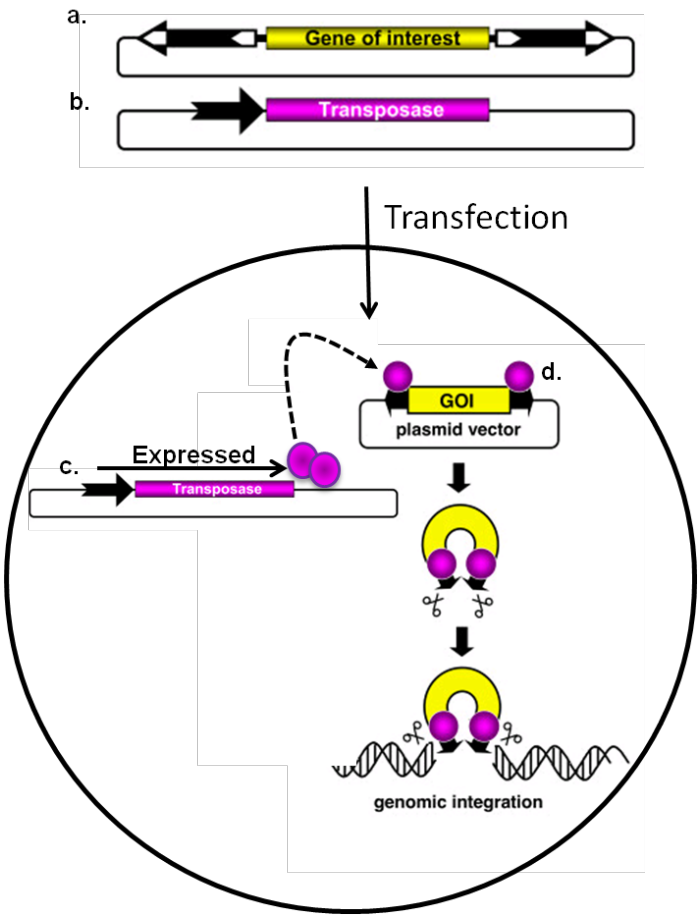


Figure 1.15 Transposable element vectors. (a) Binary system of "donor" plasmid carrying gene of interest flanked between inverted repeats and (b) "helper" plasmid carrying transposase gene. (c) the transposase is expressed in trans and binds the ITRs, (d) the gene of interest is excised and integrated into the genome. Adapted from Ivics and Izsvak 2010.

Work carried out using piggyBac-sleeping beauty vector hybrids (Wang *et al*. 2008) suggests piggyBac as a better candidate for mammalian transgenesis than sleeping beauty. Vectors were constructed by inserting a gene conferring neomycin resistance flanked by SB repeats into a piggyBac vector and then assessing transposition frequency when transfected into cells expressing either sleeping beauty or piggyBac specific transposase. The number of cells resistant to neomycin determined the transposition efficiency after transfection. The results showed significantly higher transposition efficiency in cells expressing the piggyBac transposase than those expressing the sleeping beauty.

1.6.3.3 *Tol2* vector-mediated transgenesis

The *Tol2*-vectors consist of minimal cis-regulatory sequences of 200 and 150bp within which the 12bp inverted repeat sequences (Kawakami *et al*. 2000; Urasaki *et al*. 2006). These cis elements flank the gene or cassette that is to be integrated into the genome. Unlike other transposon-derived vectors *Tol2* transposition increases with increasing amounts of transposase without any inhibitory effect from transposase overexpression (Wu *et al*. 2006). *Tol2* has the capacity to deliver foreign DNA up to 11kb in size without affect on integration frequency (Urasaki *et al*. 2006).

1.6.3.3.1 Tol2 vector-mediated transgenesis of vertebrates

Tol2-mediated transgenesis has been used effectively in several vertebrates; zebrafish, *Xenopus* and chickens and also in mammalian cell lines. In one experiment, zebrafish embryos were electroporated with a *Tol2* vector and transposase that had been optimized and it was found on average that 50% of the injected embryos were germline chimeras, and transmitted the eGFP transgene to the their offspring (Wilson *et al*. 2007). In most cases the frequency of transmission ranged from 9-50% but it was reported that in one instance 100% of the progeny of one founder fish were transgenic. It was reported that on average between five and six transposition events had occurred in each founder fish genome (Cadiñanos and Bradley 2007). *Tol2* was shown to be functional in *Xenopus* when 40% of embryos injected with *Tol2* eGFP construct and transposase mRNA, were positive for *Tol2*-

mediated integration of the marker gene (Kawakami *et al*. 2004; Hamlet *et al*. 2006). Some of the frogs produced produce GFP-expressing embryos resulting in a transmission efficiency of up to 40%. *Tol2* is also functional in mammalian cells and has been used effectively to confer neomycin resistance in mouse ES cells and human HeLa cells (Wu *et al*. 2006; Balciunus *et al*. 2006).

1.6.3.3.2 Tol2 vector-mediated transgenesis of chickens

Sato *et al*. (2005) demonstrated the efficiency of *Tol2*-derived vectors in whole embryos by integrating GFP marker genes into the genome. Using a eGFP marker attached to a CAGGs promoter (Niwa *et al*. 1991) flanked by the *Tol2* cis sequences the *Tol2* vector was tested by electroporating into chicken DF1 cells and the presomitic mesoderm (PSM) of the early embryo. The GFP marker was successfully transposed into both the DF1 cells and the cells of the PSM. In the DF1 cells, only cells co-tranfected with the transposon and the transposase plasmids expressed GFP beyond 10 days post transfection. GFP-expression persisted to at least 25 days post-transfection. The early embryos were transfected with a CAGGS plasmid expressing red fluorescent protein as a control as well as the *ol2* flanked GFP and transposase. Whilst the expression of the red fluorescence had disappeared by day (E)5 the embyos continued to express GFP until at least (E)8.5. These experiments demonstrated that the *Tol2* derived vectors were efficient at integrating DNA into chicken cells *in vitro* and *in vivo. Tol2*-derived vectors could be a useful system for chicken transgenesis.

1.6.3.4 Piggybac vector-mediated transgenesis

1.6.3.4.1 Piggybac vector-mediated transgenesis in insects

The application of piggyBac for the transgenesis of several insect species has been reported. The first piggyBac vectors used the cDNA for white-eye pigmentation to manipulate eye colour in the mediterranean fruitfly (Medfly). A piggyBac vector carrying the cDNA was co-injected with transposase into larvae (Handler *et al*. 1998). The flies produced were crossed and germline transmission of the donor eye color was observed. Transgenesis experiments in *D.melanogaster* used eye colour

manipulation in the same way as had been reported in the medfly (Handler and Harell, 1999). Using the piggyBac vector eye pigmentation was reintroduced but it was also noted that a sex-linked lethal integration may also have occurred due to eye pigmentation only being observed in females. Sex-lethality was circumvented by the replacing the promoter for the helper plasmid with the promoter for the *D.melanogaster hsp70*. This allowed for the switching on of transgene integration in the adult and circumventing the effects of possible sex-linked lethal integration. These experiments using the conditional switching on of transposition resulted in an increase from 3% to 26% in the rate of transgensis. This confirmed that piggyBac was as effective in the transgenesis of *D.melanogaster* as it is in *C.capitata* (Handler and Harell, 1998).

1.6.3.4.2 PiggyBac vector-mediated transgenesis of mammalian cells

PiggyBac-derived vectors with the transposase expressed in trans have been used successfully to transfect human, mouse and rat cells (Ding *et al*. 2005; Blair *et al*. 2011) The mouse and rat experiments showed successful stable transfection of ES and EG cell lines that had been stably transfected using piggyBac-derived vectors. These cells were successfully used in the production of germline transgenics. Transposase-mediated integration of the transgene in the mouse ES cells was confirmed by the presence of a TTAA repeat at the region of integration (Ding *et al*. 2005). The use of piggyBac in mammalian cells has not been restricted to the mouse and is functional in human ES cells (Chen *et al*. 2009), pig cells (Clark *et al*. 2007), chicken spinal chord and chicken fibroblast cells (Lu *et al*. 2009).

1.6.3.4.3 Improving piggyBac vector-mediated transgenesis of mammalian cells

Cadiñanos and Bradley (2007) manipulated the coding sequences of the transposon and transposase vectors and improved transposition in mouse ES cells. Optimisation of the piggyBac transposase for mouse codon usage was achieved by replacing each codon of the insect sequence with one that is preferentially translated in mouse cells and then inserting this sequence into an expression vector. Comparison of the insect

and mouse transposases showed that the mouse-optimized transposase was 20-fold more efficient. Investigation of piggyBac inverted terminal repeats and their effect on luciferase transgene expression indicated that the insect promoter within the 5' inverted terminal repeat was transcriptionally active in mammalian cell lines (Cadiñanos and Bradley 2007). Furthermore, a functional promoter is present in the 5' inverted terminal repeat and the 3' inverted terminal repeat acts as an enhancer (Shi *et al*. 2007). Given these results it was determined that placement of the 3' inverted terminal repeat upstream of the gene of interest could ensure expression is driven by the host's endogenous promoters and not by the piggyBac vector itself. Cadiñanos and Bradley, (2007) also successfully created inducible versions of the mouse transposase that would only express in the presence of 4- hydroxytamoxifen (4-OHT). This inducible construct makes piggyBac a more useful vector for use in cancer research and gene therapies where excess transposition resulting in genomic rearrangements could have extremely adverse results.

The published work described here outlines that piggyBac-derived vectors are functional in mammalian cells and are also functional in the chicken. PiggyBac therefore offer potential as a tool for the manipulation of cultured chicken PGCs.

1.7 THESIS OBJECTIVES

1. Using the previously published method for the isolation and propagation of chicken PGCs in culture establish lines of chicken PGCs.
2. By investigation of the signalling pathways PI3K/AKT, MEK/ERK and JAK/STAT gain better understanding of the growth factor and cytokine requirements for chicken PGC culture *in vitro* with a view to improving the culture methodology.
3. Use transposon-derived vectors to genetically modify cultured chicken PGCs and assess efficiency of the system compared to previously published data.

CHAPTER 2: MATERIALS AND METHODS

2.1 STOCK SOLUTIONS

2.1.1 General Stock Solutions

100ml 5x Creosol red

60% sucrose; 1mM creosol red. Sucrose dissolved in dH_2O on a hot plate then cooled prior to addition of the creosol red dye.

Dulbecco's solution (1L)

10 phosphate buffered saline tablets (PBS, Oxoid) were dissolved in 800 ml of dH_2O. Tablets contain: NaCl 8g/L; 0.2g/L KCL 0.2g/L; KH_2PO_4 1.15g/L; Na_2HPO_4 0.24g/L. The pH was adjusted to pH7.4 using 1M HCL and solution made up to 1L with dH_2O.

Gel Loading Dye

Bromophenol blue 0.25% w/v; Xylene cyanol FF 0.25% w/v; Ficoll Type 400 (Pharmacia) 15% w/v in dH_2O.

SOB medium

For 1L: Tryptone 20g (Fisher Scientific); yeast extract 5g; NaCl 0.5g; 10ml 250mM KCL solution (KCL, 1.86g in 100ml dH_2O). The pH was adjusted to pH7.0 using 5M NaOH. Make up to 1L in dH_2O. Sterilize by autoclaving 20 min at 15 psi (1.05 Kg/cm^2) on liquid cycle.

Luria-Bertani Agar (LB Agar)

1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.125M NaCl and 1.5% Sugar (Difco)

Phosphate buffered saline containing Tween-20 (PBT)

0.1% Tween-20 in Dulbecco's solution.

50x TAE electrophoresis buffer

For 1L: Tris (hydroxymethyl) aminomethane hydrochloride (Tris) base 242g; glacial acetic acid 57.1ml; 0.5M ethylenediaminetetraacetic acid (EDTA) solution pH8.0, 100ml; solution made up to 1L with dH_2O.

Tris/EDTA (TE)

10mM tris (hydroxymethyl) aminomethane hydrochloride (Tris/HCL), 1mM ethylenediaminetetraacetic acid (EDTA) solution.

DNA loading buffer (6x)

0.25% bromophenol blue, 0.25% xylene cyanol FF and 15% Ficoll type 400 (Pharmacia) in distilled water.

2.1.2 Stock Solutions for embedding tissue samples

0.24M Phosphate buffer pH7.2

For 3L: $NaH_2PO_4.H_2O$, 19.2g; Na_2HPO_4, 81g. The pH was adjusted to pH7.2 with 1M HCL and the solution made up to 3L using dH_2O.

0.12M Phosphate buffer /15% sucrose

0.24M Phosphate buffer was diluted by half. Sucrose was added to give final concentration of 15% (w/v). Solution stored at 4^oC.

0.12M Phosphate buffer /15% sucrose /7.5% gelatine

0.24M Phosphate buffer was diluted by half. Sucrose was added to give final concentration of 15% (w/v). Gelatine was added to give a final concentration of 7.5% (w/v). Sucrose and gelatine were dissolved by heating to 37^oC in a water bath. Solution stored in 50ml aliquots at -20^oC.

2.1.3 Stock Solutions for Western Analysis

Protein extraction solution

Detergent based extraction solution:

3% SDS; 3% Beta mercaptoethanol; 10% Glycerol; 6.25μM Tris HCL pH6.8; 0.005% Bromophenol blue.

1x Running buffer

50ml NuPage® MOPS SDS running buffer (20X, Invitrogen) in 950ml dH_2O.

10x Transfer buffer

For 1L: Tris base 58g; glycine 29.3g; 20 % sodium dodecyl sulphate (SDS), 18.8ml; made up to 1L using dH_2O.

1x Transfer buffer

For 1L: 100ml 10X transfer buffer; 200ml ethanol; made up to 1L using dH_2O, chilled to 4^oC.

25x TBS

For 2L: Tris base 125g; NaCl, 400g. The pH was adjusted to pH7.6 with 1M HCL and the solution made up to 2L using dH_2O.

1x TBST wash buffer

For 2L: 25x TBS 80ml; Tween-20 2ml. Made up to 2L using dH_2O.

Block

Marvel milk powder 2.5g; dissolved in TBST 50ml.

Primary Antibody Buffer

BSA (Fraction V, Sigma #A9647) 0.5g; TBST 10ml.

Secondary Antibody Buffer

Block 1ml; TBST 9ml.

2.1.4 Stock Solutions for Southern Analysis

Depurination Solution (0.25M HCL)

For 1L: Hydrochloric Acid (HCL) 11ml; dH_2O 989ml.

Denaturation Solution (0.5M NaOH; 1.5M NaCl)

For 1L: NaCl 87.66g; NaOH 20g; dissolved in dH_2O up to 1L.

Neutralisation Solution (1M TRIS; 1.5M NaCl; pH8.0)

For1L: Tris base 60.5g; NaCl 87.66g. The pH was adjusted to pH8.0 with 1M HCL and the solution made up to 1L using dH_2O.

Hybridisation Buffer (500mM Sodium Phosphate / 7% SDS)

For 200ml: 1M NaH_2PO_4 31.6ml; 1M Na_2HPO_4 68.4ml; 20% SDS 70ml; dH_2O 30ml. Warm at 55^0C prior to use to dissolve SDS.

Blocking agents

10mg/ml ssDNA (Herring sperm) 200µl; 10mg/ml tRNA (Salmon) 40µl.

2.1.5 Stock Solutions for Immunohistochemistry

Hoechst nuclear staining solution

BizBenzimide (Sigma) was dissolved in dH_2O to give a final concentration of 1mg/ml.

4% paraformaldehyde solution (100ml)

4g Paraformaldehyde dissolved in 0.1M Phosphate buffer. Heat to 60-65 ºC whilst stirring. 1N NaOH added drop wise until solution clears. Cool and filter.

2.1.6 Stock Solutions for cell culture

Mouse and rat fibroblast cell culture medium (500ml)

Knock-out Dulbecco's Modified Eagles Medium (KO-DMEM; GIBCO) 450ml; L-glutamine (Invitrogen) 6ml; foetal bovine serum (GIBCO) 50ml; non-essential amino acids (Invitrogen) 5ml; 1X penicillin/streptomycin (Invitrogen) 5ml.

BRL-conditioned medium (600ml)

Knock-out Dulbecco's Modified Eagles Medium (KO-DMEM; GIBCO) 558ml; foetal bovine serum (PAA) 30ml; 1X Glutamax (Invitrogen) 6ml; non-essential amino acids (Invitrogen) 6ml; 0.1mM beta-mercaptoethanol (Invitrogen). 50ml conditioned on a confluent layer of BRL cells for 3 days in a T150 flask.

Basic PGC culture medium (25ml)

Knock-out Dulbecco's Modified Eagles Medium (KO-DMEM; GIBCO) 10ml; BRL-conditioned Medium 12.3ml; foetal bovine serum (PAA) 1.9ml; chicken serum (Biosera) 625µl; 1X Glutamax (Invitrogen) 250µl; non-essential amino acids (Invitrogen) 250µl; nucleosides (Invitrogen) 250µl; 100mM sodium pyruvate (Invitrogen) 50µl; 50nM 2-mercaptoethanol (Invitrogen) 50µl; 1X penicillin/streptomycin (Invitrogen) 50µl.

Van de Lavoir culture medium (25ml)

Basic PGC culture medium plus hFGF (4ng/ml) and SCF (6ng/ml); 1X Glutamax (Invitrogen) 250µl; non-essential amino acids (Invitrogen) 250µl; nucleosides (Invitrogen) 250µl; 100mM sodium pyruvate (Invitrogen) 50µl; 50nM 2-mercaptoethanol (Invitrogen) 50µl; 1X penicillin/streptomycin (Invitrogen) 50µl.

Minimal (Starvation) medium (25ml)

Knock-out Dulbecco's Modified Eagles Medium (KO-DMEM; GIBCO)

Freezing medium

Culture medium (specific to cell line being cryopreseved) containing 20% DMSO. Filtered through a 0.1µM filter.

2.2 CENTRIFUGATION

Tubes used	Temperature	Centrifuge	Rotor
PCR strip tubes	Room temperature	Tupe-Strip Picofuge™ (Stratagene)	-
0.5ml / 1.5ml	Room temperature	Mini spin plus bench top centrifuge (Eppendorf)	-
0.5ml / 1.5ml	4°C	Biofuge fresco centrifuge (Heraeus)	-
1.5ml	Room temperature	Biofuge pico (Heraeus)	-
15ml	Room temperature	GS-15R (Beckman)	S4180 (Beckman)
15ml	Room temperature	Function line (Heraeus)	#8172 (Heraeus)
15ml-50ml	Room temperature	B4i (Jouan)	-
>50ml	4°C	Sorvall® RC-5B refrigerated superspeed centrifuge (Du Pont Instuments	Sorvall® SLA-1500 Super-Lite® rotar

2.3 AGAROSE GEL ELECTROPHORESIS OF DNA

Molecular biology grade agarose was weighed to give final concentration of 0.9% - 2% (w/v), depending on predicted band size, and dissolved in 1 x TAE electrophoresis buffer by heating in a microwave and allowed to cool to hand hot. For ethidium bromide gels: ethidium bromide (Sigma) was added to a final concentration of 0.5µg/ml before pouring the gel using a horizontal gel electrophoresis gel kit. For Sybersafe gels: 1µl/ml of Sybersafe gel dye was added before pouring. DNA samples were mixed with 6X loading buffer prior to loading onto the gel. 2µl of TrackIt™ 1kb plus DNA ladder (Invitrogen) was used as a size marker. The Gel Logic 200 imaging system (Kodak) was used to visualise the gels.

2.4 FROZEN SECTIONS FOR IMMUNOHISTOCHEMISTRY

2.4.1 Sample pre-treatment

2.4.1.1 Sample fixation

Embryonic tissues were fixed in 4% paraformaldehyde for 30 min at room temperature. Adult tissues were fixed in 4% paraformaldehyde overnight at 4°C.

Tissues were rinsed three times in PBS and transferred to stock 0.12M phosphate buffer/15% sucrose solution and incubated overnight at 4^{o}C.

2.4.1.2 Embedding tissue samples in gelatine

0.12M Phosphate buffer/15% sucrose/7.5% gelatine stock solution was thawed at 37^{o}C for 2 hours. A 'gelatine bed' was made by adding 3ml 0.12M Phosphate buffer/15% sucrose/7.5% gelatine to a small plastic weighing tray and left to set at room temperature for 30 min. The tissue samples to be embedded were incubated in 1ml of 0.12M Phosphate buffer/15% sucrose/7.5% gelatine stock solution 37^{o}C for 30 min. The tissue samples were poured onto the gelatine bed and positioned under a microscope. 0.12M Phosphate buffer/15% sucrose/7.5% gelatine stock solution was added to cover the tissue and left to set at 4^{0}C for 15 min. Samples were cut from the gelatine bed as blocks and mounted onto card with OCT (Bright Instruments Co. Ltd). Isopentane was chilled on dry ice to a temperature of -65^{o}C. The gelatine blocks were frozen in the chilled isopentane and stored at -80^{o}C until sectioned.

2.4.1.3 Sectioning of frozen material

An OTF5000/HS-001 cryostat with solid knife block holder (Bright Instruments) was used. The cryostat chamber was set at -18^{o}C and the sample temperature at -21^{o}C. Samples were transported and maintained on dry ice. Samples were mounted on the chuck using OCT. The block was left to equilibrate -21^{o}C to before sections were cut at 15-20µm. Sections were collected on polylysine slides (VWR international) and stored at -20^{o}C until use.

2.4.2 Protein detection on frozen sections

2.4.2.1 Preparation of frozen sections for protein detection

To remove the remains of the sucrose-gelatine block, frozen slides of sections were incubated at 37^{o}C in PBS for 30 min and air-dried. The sections were washed five times in 1ml PBS. Sections were incubated at room temperature for 20 min in 1ml antibody blocking serum. Primary antibody/antibodies (Table 2.1) were diluted, as appropriate in PBS and 1ml was added to sections. All further steps were carried out

ANTIBODY	TYPE	DILUTION	APPLICATION	SUPPLIER
pAKT	1°	1/1000	WB	CST
panAKT	1°	1/1000	WB	CST
pSTAT3 (TYR705)	1°	1/1000	WB	CST
STAT3	1°	1/1000	WB	CST
pERK1/2	1°	1/1000	WB	CST
ERK2	1°	1/1000	WB	BD Transduction Laboratories
γ-Tubulin	1°	1/500	WB	SIGMA
anti-rabbit IgG-HRP	2°	1/1000	WB	CST
anti-mouse IgG-HRP	2°	1/1000	WB	CST
Anti-GFP-rabbit-IgG Alexa Fluor® 488 conjugate	Conjugated	1/500	IH	Invitrogen

Table 2.1 Table of antibodies. WB: Western Blot; IH: Immunohistochemistry.

in a humidity chamber until mounting. The sections were incubated overnight at 4°C. Sections were rinsed three times, quickly, in 1ml PBS and incubated for one hour in 1ml PBS. The PBS was changed every 20 min. The sections were incubated at room temperature for one hour in 1ml secondary antibody (Table 2.1). Sections were rinsed three times, quickly, in 1ml PBS and incubated for one hour in 1ml PBS. Hoechst nuclear staining (2.5.1) was added to PBS to give a 1/100 dilution. 1ml was

added to sections and incubated for 1 min. Sections were rinsed three times, quickly, in 1ml PBS and mounted using Hydromount (National Diagnostics). Sections were viewed under a microscope.

2.4.2.2 GFP staining of frozen sections

Primary antibody solution was made up by making a 1/500 dilution of anti-green fluorescent protein; IgG fractions, Alexa Fluor conjugate antibody (Invitrogen) (Table 2.1) in PBS and 15µl/ml of donkey serum. The sections were incubated overnight at 4^{o}C in the antibody in a humidity chamber. GFP positive cells were visible when illuminated by UV light transmitted through a FIT-C filter.

2.5 ANIMAL METHODS

2.5.1 Source of chicken embryonic and adult tissue

Chicken embryos and adult material were obtained from the Roslin Institute flocks of ISA brown layer birds both wild type and GFP+ (McGrew et al., 2008).

2.5.2 Staging chicken embryos

Embryos were staged using the method outline by Hamburger & Hamilton 1951. The stages are referred to as stage "X" HH throughout this thesis.

2.5.3 Aspiration of blood from stage 14-16 H&H embryos

Fertile eggs were incubated for 2.5 days prior to aspiration. Micro-capillary tubes (Harvard Apparatus) with a 1.5mm external and 1.17mm internal diameter were made into glass needles using a moving-coil microelectrode puller, model 753 (Campden Instruments Ltd). Using a MB3-T turbo microbeveller (Research Instruments Ltd) the needles were bevelled to create an aperture of 20-30µm and UV sterilised prior to use. Sharp scissors were used to make a hole in the shell of an egg at the blunt end. The embryo was visualised by cutting a window in the eggshell. Using the glass needles 1-5µl of blood was aspirated from the embryo and dispensed into culture medium.

2.5.4 Injection of single cell PGC suspension into stage 14-16 H&H embryos

2.5.4.1 Preparation of PGC cells

0.5 – 1.5ml of PGC in suspension were placed in a 1.5ml screw cap microfuge tube. The cells were centrifuged at 1000 rpm for 3 min. The supernatant was removed by aspiration using a fine glass capillary. The PGC pellet was resuspended in the required amount of medium. 1µl of FAS-green dye was added to the suspension immediately prior to injection.

2.5.4.2. Injection of PGCs into the vascular system

Wild type host embryos were incubated prior to injection for 2.5 days. Glass needles were made as described (2.5.3). The embryo was visualised by windowing (2.5.3) or by opening egg and placing embryo in a low sided cling film covered curved bottom dish (Figure 2.1) PGC suspension was taken up into a glass capillary and injected into either the aorta or developing heart of the host embryo (Figure 2.1a). Windowed embryos were resealed with tape whilst embryos injected in the curved bottom dish were cultured to hatch using phase III of the *ex vivo* culture system (Perry 1988; Figure 2.1b).

2.5.5 Dissection of embryos at stages 26-28 and stages 33-35 H&H

An egg was cracked open and the embryo was transferred from the eggshell to a petri dish containing Dulbecco's solution. The head and vitelline membranes were removed and the embryo's abdominal region was opened and the gut removed. For removal of the gonads from the embryo carcass, a 10Å scalpel (Swann-Morton) was slid beneath the mesonephros and moved back and forth to cut connective tissue. The excess mesonephros tissue was cut away using 5mm spring scissors.

2.5.6 Preparation of chicken embryonic fibroblasts (CEFs)

Freshly laid fertilised eggs were incubated at 37^{o}C for 9 days. In a sterile laminar flow hood the eggs were opened and the embryo decapitated with scissors. The embryo was placed in a sterile petri dish and using forceps the body wall was cut

open on the ventral surface. The viscera were removed and discarded and the rest of the carcass finely minced with scissors. The homogenate was transferred to a sterile 15ml centrifuge tube, 3ml of trypsin added and left at room temperature for 15 min, pipetting up and down every 5 min. 3ml of complete medium: DMEM, Invitrogen; 5% FBS (PAA), Pen/Strep (Invitrogen), Glutamine (Invitrogen) was added to inactivate the trypsin. The dissociated embryonic cells were plated in 100mm tissue culture dishes and incubated at 37 ^{o}C, 5% CO_2. After 24 hours medium was removed and replaced with fresh complete medium.

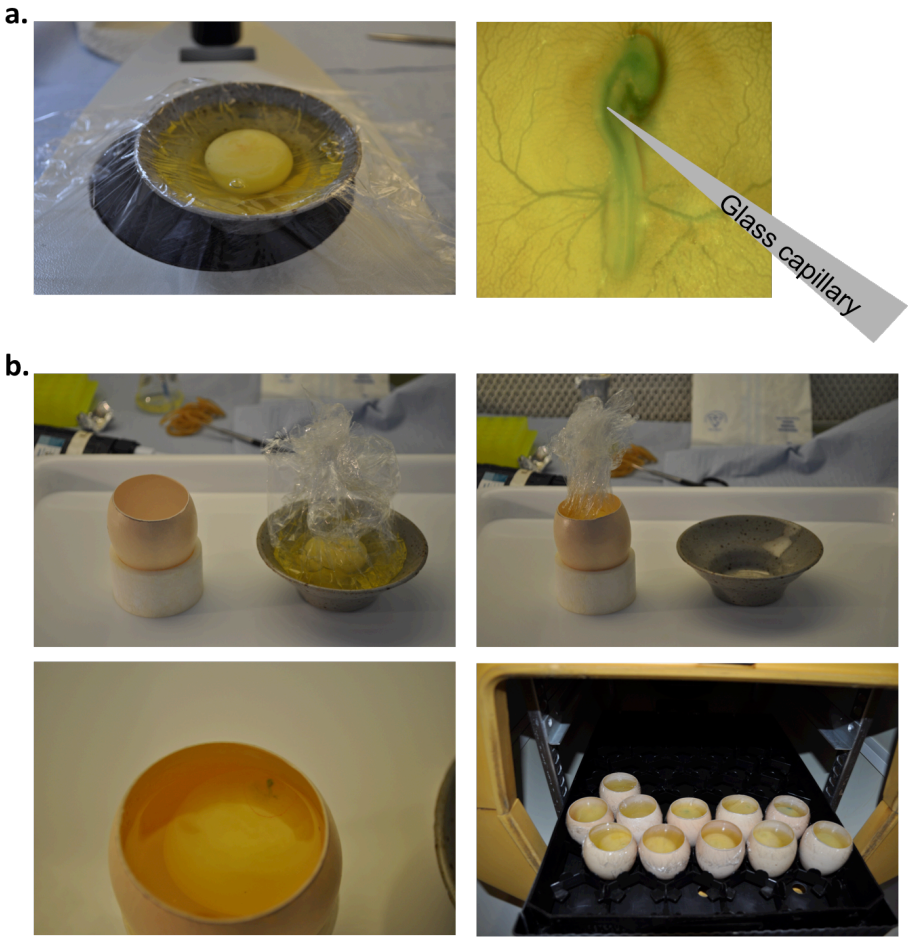


Figure 2.1 Injecting cultured PGCs in to host embryos and culturing in phase 3 shells. (a) Host embryos are removed from their shells and placed in a shallow dish lined with saran wrap. The PGCs are then injected using a glass needle, into the developing hart or aorta. (b) The injected embryo is then transferred from the dish into a surrogate shell and cultured to hatch using phase III of the *ex vivo* culture system (Perry 1988).

2.6 MOLECULAR BIOLOGICAL METHODS

2.6.1 Purification of DNA fragments from agarose gels

DNA fragments were purified using Perfectprep® Gel Cleanup kit (Eppendorf). The desired ethidium bromide stained DNA fragment was excised from a TAE agarose gel (up to 4% agarose). The gel slice was weighed in a microcentrifuge tube (maximum of 400mg per tube). Three volumes of binding buffer were added for every one volume of gel slice (1mg of weight equals 1µl of volume). The sample was incubated at 50ºC for 5 to 10 min in a heat block, vortexing every 2 to 3 min. Once the gel slice was completely dissolved one volume of isopropanol (equal to the original gel slice volume) was added and mixed by inversion. Up to 800µl of sample was applied to a spin column in collection tube and centrifuged at 6,000 - 10,000 x g for 1 min. The filtrate was discarded and the spin column placed back into the same collection tube. If the sample volume was greater than 800µl, the spin column was reloaded and spun again. 750µl of wash buffer was added to the spin column and centrifuged at 6,000 - 10,000 x g for 1 min. The filtrate was discarded and the spin column placed back into the same collection tube. To remove any residual wash buffer the column was centrifuged at 6,000 - 10,000 x g for one min. The spin column was placed in a new collection tube and 30µl of elution buffer applied. The spin column was centrifuged at 6,000 - 10,000 x g for 1 min. The eluted DNA sample was transferred to a sterile, labelled 1.5ml Microfuge tube and stored at -20^{o}C.

2.6.2 Transformation of bacteria

2.6.2.1 Ultracompetent *E.coli* XL 10-Gold cells (Stratagene)

Cells were thawed slowly on ice. 100µl of cells and 4µl of β-mercaptoethanol were aliquoted into 2 pre-chilled 12ml BD Falcon polypropylene round-bottomed tubes. The cells were mixed gently and incubated on ice for 10 min. 0.1-50ng of DNA (2µl ligation mixture) was added to one tube and 1µl of 1/10 dilution of positive control DNA (pUC18) to the other and mixed. Tubes were incubated on ice for 30 min prior to being heat shocked in a 42^{o}C water bath for 45 sec. Tubes were chilled on ice for 2 min before addition of 900µl of pre-warmed SOC (42^{o}C) to each tube. Tubes were

incubated, shaking at 250 rpm, for 1 hour. 250µl of mix was spread on two separate plates of LB-agar containing 100µg/ml ampicillin and incubated overnight at 37^{o}C.

2.6.2.2 DH5 alpha cells

Cells were thawed slowly on ice. 50µl of cells and 1µl of DNA (max 100ng) or 10pg pUC19 control DNA were incubated, in 12ml BD Falcon polypropylene round-bottomed tubes, on ice for 20 min. The bacterial/DNA suspensions were heat-shocked in a 42^{o}C water bath for 2 min exactly. Tubes were chilled on ice for 2 min followed by addition of 1ml of SOB to each tube. Tubes were incubated, shaking at 250 rpm, for 1 hour. The cells were transferred into a 1.5ml microfuge tube and centrifuged for 2 min at full-speed. The supernatant was aspirated leaving approximately 300µl of medium behind. Cells were resuspended in the residual medium. 200µl of cells was spread on LB-agar containing 100µg/ml ampicillin plus 100µl XGAL (50mg/ml) and 125µl 1M IPTG added for blue/white screening for identification of recombinant plasmids. The plates were incubated overnight at 37^{o}C.

2.6.3 Preparation and sequencing of plasmid DNA

2.6.3.1 Small-scale preparation of plasmid DNA

DNA was isolated using the Wizard *Plus* SV Minipreps system (Promega). A single colony from a fresh LB agar plate containing the appropriate antibiotic was used to inoculate 3ml of LB agar (with antibiotic) in a 15ml centrifuge tube. The culture was incubated overnight, at 37^{o}C and shaking at 250 rpm. Cultures were harvested by centrifugation (10000 x g for 5 min) and the supernatant discarded. Cells were resuspended in 250µl of cell resuspension solution and transferred to a 1.5ml microcentrifuge tube. 250µl of cell lysis solution was added and mixed by inverting 4 times, tubes were left to incubate until the lysate began to clear but no longer than 5 min. 10µl of alkaline protease was added and the tubes incubated at room temperature for 5 min. 350µl of neutralisation solution was added and mixed immediately by inverting the tubes 4 times. The bacterial lysate was centrifuged (14000 x g for 10 min) at room temperature. The cleared lysate (approximately 850µl) was transferred to a spin column in a collection tube and centrifuged (14000 x

g for 1 min, room temperature) and the supernatant discarded. 750µl of column wash solution was added to the column which was centrifuged (14000 x g for 1 min, room temperature) and supernatant discarded. This step was repeated with 250µl of wash solution and a 2 min centrifugation. After discarding the flow through the columns were centrifuged (14000 x g for 1 min, room temperature) before elution of DNA into a clean collection tube by the addition of 100µl of dH_2O and centrifugation (14000 x g for 1 min, room temperature).

2.6.3.2 Large-scale preparation of plasmid DNA

DNA was isolated using the PureLink™ HiPure Plasmid Filter Purification Kit (Invitrogen). A single colony from a fresh LB agar plate containing the appropriate antibiotic was used to inoculate a 5ml starter culture of LB medium (with antibiotic) in a 15ml polypropylene tube. This culture was incubated for 8 hours at 37^{o}C whilst shaking at 250 rpm. The starter culture was used to inoculate 250ml of LB medium (with antibiotic) and grown for 12-16 hours at 37^{o}C, shaking at 250 rpm.

Preparation of cell lysate

Cells were harvested by centrifuging the overnight LB-culture at 4,000 x g for 10 min and all supernatant discarded. Cells were resuspended in 10ml resuspension buffer (R3) with RNase A (20ml for culture volumes >200ml) and the suspension transferred to a 50ml centrifuge tube. 10ml of lysis buffer (L7) (20ml for culture volumes >200ml) was added and mixed gently by inversion five times. The homogeneous lysate mixture was incubated at room temperature for 5 min. 10ml precipitation buffer (N3) (20ml for culture volumes >200ml) was added and the tubes mixed immediately by inversion.

Loading filter column and washing DNA

The precipitated lysate including all the precipitated material was transferred into the equilibrated HiPure Filter Midi Column. The lysate was allowed to flow through the filter by gravity until the flow stopped (10-15 min) or became very slow (<1 drop per 10 seconds). The flow through was discarded. In order to increase the final DNA yield the residual bacterial lysate in the HiPure Filter Maxi column was washed out, by gravity flow until the flow stopped or dripping became very slow, with 10ml

Wash Buffer (W8). Immediately after the HiPure Filter Midi Column had stopped dripping, the inner Filtration Cartridge from the column was removed and discarded. The maxi column was washed with 50ml of wash buffer (W8) by gravity flow and the flow-through discarded.

DNA elution and storage

DNA was eluted using 15ml Elution Buffer (E4), by gravity flow, into a sterile 50ml centrifuge tube (elution tube) and the column discarded. 10.5ml isopropanol was added to the elution tube, which was mixed well. The tube was centrifuged at >15,000 x g for 30 min at 4°C and the supernatant carefully discarded. The DNA pellet was dissolved in 5ml 70% ethanol and the tube centrifuged at >15,000 x g for 5 min at 4°C. Again the supernatant was carefully discarded. The pellet was left to air dry for approximately 10 min and the DNA redissolved in 200-500µl of TE buffer depending on concentration. If insoluble particles were present the DNA was centrifuged at high speed at room temperature for 1 min and the supernatant (DNA sample) transferred to a fresh tube. The purified DNA was stored at -20°C or used immediately for the desired downstream application.

2.6.3.3 Sequencing of plasmid DNA

Sequencing was carried out by the University of Dundee Sequencing service. 200-300ng of DNA in 15µl dH_2O per reaction and the appropriate primers were sent to The Sequencing Service. DNA was sequenced for both strands was determined.

2.6.4 Isolation and quantification of RNA from tissues/cells

2.6.4.1 RNA extraction using RNA-Bee

All equipment was autoclaved twice or wiped down thoroughly with RNase Zap before use. Samples were kept on ice throughout the procedure. Tissue or cells were placed in a plastic universal tube (Bibby Sterilin) with approximately 2ml RNA – Bee/100mg of tissue/cells. Tissue/cells were homogenised using a polytron PT 2100 cleaned prior to use with cold 0.2M NaOH and double autoclaved dH_2O. The homogenised tissue/cells were placed on ice, 0.1 volume of chloroform added and the sample shaken vigorously for 30 sec. The sample was incubated for 5 min on ice

and centrifuged at 4°C, for 20 min at 12000 x g. The upper aqueous phase was transferred to a new tube, being careful to avoid protein contamination by interphase carry over. One volume of cold isopropanol was added to the sample and mixed well before incubation on ice for 15 min. The sample was centrifuged (12000 x g; 20 min; 4°C) and the supernatant removed. The pellet was washed in 85% ethanol and centrifuged (7500 x g; 10 min; 4°C) and ethanol removed. Pellet was air dried on ice or by incubating in a heat block for 2 min at 45°C before solubilisation in double autoclaved dH_2O. To avoid degradation RNA was stored at -80°C as an ethanol precipitated by adding 0.1 volumes of 3M sodium acetate (NaOAc) and 2.5 volumes of absolute ethanol and mixing.

2.6.4.2 RNA extraction using RNeasy minikit (Qiagen)

Total RNA was isolated from cells using RNeasy minikit (Qiagen). Cells were harvested according to the manufacturer's guidelines. Cells (> 5 x 10^6) were collected and pelleted by centrifugation, 3 min at 1000 rpm in a 1.5ml screw cap microfuge tube and the supernatant removed. 350µl (600µl for larger cell pellets) of buffer RLT was added to the tubes and mixed by vortexing to lyse the cells. The cell lysate was added directly onto a QIA shredder spin column in a 2ml collection tube and centrifuged for 2 min at full speed. One volume (350µl) of 70% ethanol was added to the homogenised lysate and mixed by pipetting. The sample including any precipitate was transferred into an RNeasy spin column, placed in a 2ml collection tube, and centrifuged for 15 s at ≥10,000 rpm. The flow through was discarded. 700µl of buffer RW1 was added to the spin column, centrifuged for 15 s at ≥10,000 rpm and the flow through discarded. 500µl of buffer RPE was added to the spin column, centrifuged for 2 min at ≥10,000 rpm. The flow through was discarded and the spin column centrifuged for a further 1 min at ≥10,000 rpm to dry the column. The column was placed in a sterile 1.5ml collection and 50µl of RNase free water added to the column membrane. The column was centrifuged for 1 min ≥10,000 rpm to elute the RNA into the collection tube. The RNA was quantified by nanodrop and stored at -80°C.

2.6.4.3 Analysing RNA quality

The Agilent 2100 Bioanalyzer was used to analyse RNA quality. Sample RNA (200ng) and RNA 6000 ladder (Ambion) were denatured at 70^{o}C for two min and stored on ice. The RNA Nano chip was loaded following the manufacturers guidelines. The chip containing the RNA samples was loaded in the Agilent 2100 Bioanalyzer and the Eukaryote total RNA Nano program selected for sample analysis.

Preparation of gel for use with the Agilent 2100 Bioanalyzer

Reagents were equilibrated to room temperature for thirty min before use. 550µl of RNA 6000 Nano gel matrix was added to a spin filter provided and centrifuged for ten min at 1500 x g at room temperature. The gel was stored in 65µl aliquots at 4^{o}C.

Preparation of gel-dye mix for use with the Agilent 2100 Bioanalyzer

Reagents were equilibrated to room temperature for 30 min before use. 550µl of RNA 6000 Nano dye was vortexed for 10 sec and spun down. 1µl of dye was added to 65µl of filtered gel and the solution vortexed. The tube was centrifuged at 13000 x g for 10 min at room temperature.

2.6.5 cDNA synthesis

The Promega Reverse Transcription System was used. 1µg of RNA was heat-treated at 70^{o}C for 10 min. The following 20µl reaction mix was made up: 25mM $MgCl_2$, 4µl; 10x reverse transcription buffer, 2µl; 10mM dNTP mixture, 2µl; recombinant RNasin, 0.75µl; random primers, 0.5µl; total RNA, 1µg. The samples were incubated at room temperature for 10 min, followed by 42^{o}C for 55 min, 95^{o}C for 5 min and cooled on ice. cDNA was stored at -20^{o}C. For negative control samples the above reactions were carried out without AMV Reverse Transcriptase.

2.6.6 Reverse Transcriptase PCR

The following 15µl reaction mixture was made up: dH_2O, 6µl; 10x buffer + $MgCl_2$ (Roche), 1.5µl; 10mM dNTPs (Invitrogen), 0.3µl; 5' primer (50pmol/µl), 0.3µl; 3' primer (50pmol/µl), 0.3µl; Fast Start Taq (Roche), 0.1 µl; 5x creosol red, 3.0 µl; sample cDNA 2 µl. Reaction mixture was vortexed and centrifuged. Master mix

without cDNA was made up for each primer set and pipetted into PCR tube strips (Abgene). After addition of the cDNA, the tubes were centrifuged and loaded into a JMBS 0.2G (Hybaid) PCR machine. The following PCR reaction was carried out: Stage 1 (1 cycle), 95^{o}C for 20 min; Stage 2 (30 cycles) step 1, 95^{o}C for 30 sec; step 2, annealing temperature for 30 sec; step 3, 72^{o}C for 1 min; Stage 3 (1 cycle), 60^{o}C for 30 min; hold at 4^{o}C. Samples were resolved on a 0.9% TAE agarose gel. Primers are listed in table 2.2.

2.6.7 DNA Purification Protocol For 4 µl Avian Whole Blood or 8 µl Semen

Cell Lysis

Use fresh blood within 10 seconds of venipuncture to avoid clotting. Add 4 µl whole blood, or 8ml semen to a 1.5 ml tube containing 600 µl *Cell Lysis Solution***.** Quickly pipet up and down 3-5 times to lyse the cells. Usually no incubation is required; however, if cell clumps are visible after mixing, incubate at 37°C until the solution is homogeneous. Samples are stable in *Cell Lysis Solution* for at least 2 years at room temperature.

RNase Treatment

Add 1 µl RNase A Solution 30 mg/ml to the cell lysate. Mix the sample by inverting the tube 25 times and incubate at 37°C for 30 minutes.

Protein Precipitation

Cool sample to room temperature on ice. Add 200 µl Protein Precipitation Solution to the RNase A-treated cell lysate. Vortex vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution uniformly with the cell lysate. Centrifuge at 13,000-16,000 x g for 3 minutes. The precipitated proteins will form a tight pellet. If the protein pellet is not visible or tight, repeat vortex followed by incubation on ice for 5 minutes, then repeat and centrifugation.

DNA Precipitation

Transfer the supernatant containing the DNA (leaving behind the precipitated protein pellet) into a 1.5 ml centrifuge tube containing 600 µl 100%Isopropanol (2-propanol) using a fine tip pastette. Mix the sample by inverting gently 20-30 times. Centrifuge

at 13,000-16,000 x g for 1 minute; the DNA will be visible as a small white pellet. Remove the supernatant and drain tube on clean absorbent paper. Add 600 µl 70% ethanol and invert the tube several times to wash the DNA pellet. Centrifuge at 13,000 x g for 2 minutes. Carefully remove ethanol using a fine tip pastette. Pellet may be loose so watch the pellet and be careful. Invert and drain the tube on clean absorbent paper and allow to air-dry 10-30 minutes. Take care to ensure there is no ethanol remaining.

DNA Hydration

Add 100 µl DNA Hydration Solution or less if considered appropriate. Rehydrate DNA by incubating sample 1 hour at 65ºC and/or overnight at room temperature. If possible, tap tube periodically to aid in dispersing the DNA. Store DNA at 4°C. For long-term storage, store at -20°C or -80°C.

2.6.8 Semi-quantitative PCR

Prepare genomic DNA from tissue sample as per 2.6.7. Dilute the DNA to 25ng/ml. Each PCR reaction will receive 2ml (50ng) of diluted DNA prep. Set up PCR Reaction in the flow hood. The final reaction volume is 15ml, each having 2ml of sample, and 13ml of master mix. Use 8 x 0.2ml microstripes (Abgene). Always use plugged (Art) tips when setting up PCR reactions. Also only use bottled water (Sigma), which has been opened in the hood.

Make a master mix based on the following volumes:

dH_2O	7.5 ml
5X Cresol Red	3.0 ml
10 X Reaction buffer + Mg^{++}	1.5 ml
dNTP Mix (10mm each)	0.3 ml
forward primer (50pmol/ ml)	0.3 ml
reverse primer (50pmol/ ml	0.3 ml
FastStart Taq DNA polymerase	0.1 ml
	Total: 13.0 ml

Gene	Primers: Forward (F) Reverse (R)	Annealing temperature	Predicted product size	Chapter
CVH	F: AGCACAGGTGGTGAACGAACCA R: TCCAGGCCTCTTGATGCTACCGA	58°C	536bp	3
cPouV	F: TCAATGAGGCAGAGAACACG R: TCACACATTTGCGGAAGAAG	58°C	162bp	3
Sox2	F: AGGCTATGGGATGATGCAAG R: GTAGGTAGGCGATCCGTTCA	50°C	163bp	3
cMyc	F: GCACAGAGTCCAGCACAGAA R: GTTCGCCTCTTGTCGTTCTC	50°C	247bp	3
Klf4	F: AGCTCTCATCTCAAGGCACA R: GGAAAGATCCACTGCTTCCA	50°C	130bp	3
Nanog	F: TTGGAAAAGGTGGAACAAGC R: GGTGCTCTGGAAGCTGTAGG	60°C	187bp	3
CXCR4	F: AAGAGGAGGTCAGCCACAGA R: TTTCAACCGGATCTTCTTGC	50°C	150bp	3
CXCR7	F: GCTTCCTTTGCTCCACAGAC R: TTTTGGCTTGGAGATTGACC	50°C	450bp	3
Gapdh	F: CAGATCAGTTTCTATCAGC R: TGTGACTTCAATGGTGACA	58°C	343bp	3
B-actin	F: GAAGATCCTGACCGAGCGTG R: ACTGTGTTGGCATAGAGGTC	58°C	323bp	3
LEN	F: CGAGATCCTACAGTTGGCGCCCGAACAG R:ACCAGTAGTTAATTTCTGAGACCCTTGTA	58°C	250bp	3
GFP	F: ACGTAAACGGCCACAAGTTC R:AAGTCGTGCTGCTTCATGTG	58°C	175bp	5
W-PCR	F: GAAATGAATTATTTTCTGGCGAC R: CCCAAATATAACACGCTTCACT	60°C	450bp	3/5

PCR conditions: 95°C for 20 minutes; 30 cycles of 95°C for 30s, annealing temperature for 30s and 72°C for I minute, and final extension of 60°C for 30 minutes.

Table 2.2 PCR primer sequences. Forward and reverse primers are listed for all genes listed in column 1. Annealing temperatures differed between genes and are listed in column 3. The expected product size and chapter used are columns 4 and 5 respectively. The overall cycling conditions did not change and are outlined below the table.

Pipette 13ml into each tube in the strip. Ensure you have clearly numbered the first and last tube of each strip. Add 2 ml of diluted DNA sample. For the 100, 10, 1, and 0 controls add 50ng of control, non-transgenic DNA from blood.

Positive controls:

These are designed to imitate the presence of a plasmid sequence as a single copy in genomic DNA at levels of 1 copy /cell (100%), 1 copy / 10 cells (10%), and 1 copy / 100 cells (1%). For example PB-CGIP plasmid is 9kb. Chicken genome is 10^6kb. For example, in 1 mg of genomic DNA there is 9 x 10^{-6} of PB-CGIP i.e. 9pg. **Plasmid controls are diluted from stock, and always in a separate area from where the reactions are set up.**

The following 1:20 dilution is to simulate % equivalence in 50ng of genomic DNA.

e.g. PB-CGIP 9ng/ml (stock)

2µl into 198µl TE (⇨A)

↳ 10µl A into 190µl TE (⇨B)

↳ 10µl B into 90µl TE (⇨C) ⇨ **100%**

↳ 10µl C into 90µl TE (⇨D) ⇨ **10%**

↳ 10µl D into 90µl TE (⇨E) ⇨ **1%**

Analyse the samples by loading 6 ml of each reaction on a 1.2% agarose gel.

2.6.9 Restriction enzyme digests

Restriction enzymes were obtained from either Roche or New England Biolabs. Plasmid DNA was digested with 5U of restriction enzyme for each 1µg of DNA. 10X buffer supplied with enzyme was added to final volume of 1X. For some enzymes, 100µg/ml BSA was added to the digestion, to achieve 100% activity. Digests were incubated at 37^{o}C for one hour or until digestion was complete.

2.6.10 Ligation into pGEM®-T Easy vector using 2X rapid ligation buffer

The pGEM®-T Easy vector and control insert were centrifuged briefly. The 2X rapid ligation buffer was vortexed vigorously and the ligation reactions set up as follows:

2X Rapid ligation buffer	10µl	10µl	10µl

pGEM®-T Easy vector (50ng)	2µl	2µl	2µl
DNA/PCR product	Xµl	-	-
Control insert DNA	-	-	-
T4 DNA ligase (3 weiss units/µl)	1µl	1µl	1µl
ddH_2O to final volume	10µl	10µl	10µl

2.6.11 Western Analysis

2.6.11.1 Protein sample preparation

PGC suspensions were placed in a 1.5ml screw cap microfuge tube and centrifuged for 3 min at 1000 rpm. The supernatant was removed and the cells resuspended in 100µl medium and 100µl lysis buffer, mixed by inversion and stored at -20^{o}C.

2.6.11.2 Western Blotting

Sample Preparation

Protein samples stored at -20^{o}C were thawed in a heat block at 37^{o}C, sonicated for 8min in a Misonix Sonicator Ultrasonic Processor XL, boiled for 5 min at 95^{o}C and vortexed briefly. Samples were centrifuged 13000 x g for 15s, vortexed and centrifuged again before being placed on ice.

SDS polyacrylamide gel electrophoresis

Protein lysates in loading buffer (8µl) and molecular weight (MW) marker were loaded onto a precast Tris-glycine gel (Invitrogen) using the X-cell surelock mini-cell apparatus (Invitrogen) filled with running buffer. Wells not in use were filled with 1x SDS buffer. The gel was run at 50V until the MW markers began to resolve and at 100V until the dye front had reached the bottom of the gel.

Western transfer of proteins to PVDF membrane

A sheet of Hybond PVDF membrane was soaked 10 min in dH_2O and 10 min in cold 1X transfer buffer. The electroblotting transfer apparatus was assembled as illustrated in figure 2.2. The transfer was run at 90V for 75 min. The apparatus was dismantled, the membrane removed, marked for orientation and washed in PBS for 2 min, with agitation.

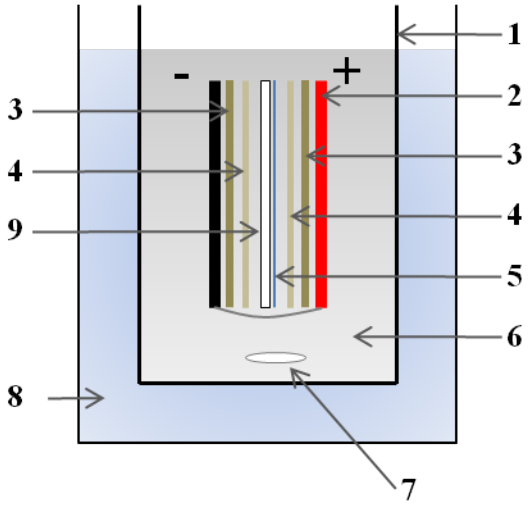


Figure 2.2 Apparatus used for wet transfer of proteins to Hybond-N membrane. (1) Electrophoresis tank, (2) transfer cassette, (3) fibre pads, (4) 3MM blotting paper, (5) Hybond-N membrane, (6) 1X cold transfer buffer (see stock solutions), (7) magnetic stirrer, (8) ice, (9) gel. The contents of the transfer cassette were assembled in a tray containing transfer buffer, to minimise bubbles that could interfere with transfer, before being inserted into the electrophoresis tank.

Detection of target proteins by chemiluminesence

The membrane was blocked overnight at 4^{o}C or 1hr at room temperature with agitation in freshly prepared blocking solution. The membrane was washed briefly in 10ml TBST. Primary antibody was diluted to the desired concentration in 5%BSA/TBST. The membrane was incubated overnight at 4^{o}C or 1hr at room temperature with agitation, in 10ml-diluted antibody. The membrane was rinsed briefly in 10ml TBST followed by three 15 min washes in TBST with agitation. The membrane was incubated, for 1 hour with agitation, in 10ml of the appropriate HRP-conjugated secondary antibody (Table 2.1), diluted in 10% block/TBST to the desired concentration. The choice of secondary antibody (e.g. anti-mouse-IgG-HRp or anti-rabbit-IgG-HRP) was dependant on the species in which the primary antibody was raised. The secondary antibody was discarded and the membrane was rinsed briefly in TBST followed by three 15 min washes in TBST with agitation. ECL western detection reagents (Invitrogen) were mixed at a ratio of 1:1 and poured on to the membrane which was incubated for 1 min at room temperature. Excess ECL

reagent was removed by blotting the membrane on tissue paper. The membrane was placed between sheets of clear plastic and exposed to Hyperfilm ECL (Amersham). Exposure times varied, depending on the protein being probed, from 15 sec to overnight. Film was developed in a Konica SRX-101A X-ograph machine.

Removal of bound antibodies from PVDF membrane

The membrane was washed in TBST for 5 min and in stripping buffer (Restore striping buffer, Thermo Scientific, CAT#21059) for 15 min, followed by two further 5 min washes in TBST.

2.6.12 Southern Analysis

2.6.12.1 Digestion of chicken genomic DNA

In a 0.5ml microfuge tube 8-10μg of DNA was added to digestion master mix; dH_2O, 10X buffer, enzyme and BSA if required; in a total volume of 50μl, using 30U enzyme (but not more than 5μl) for each digest. Digests were incubated at 37^oC overnight.

2.6.12.2 Southern Blotting

Agarose gel electrophoresis

Digested DNA preparations were run overnight at approximately 1-3 V/cm on a 0.9% agarose / TAE gel. W/V % of the gel can be altered to resolve DNA of different sizes (Table 2.3). The gel was rinsed with distilled water and photographed with a fluorescent ruler alongside (0 marker positioned at the wells). The gel was trimmed, measured and the top right hand corner cut off for orientation. At room temperature the gel was washed, with agitation, in depurination solution for 15 min, or until the bromophenol blue band had turned yellow. The gel was rinsed in dH_2O. The gel was washed, with agitation, in denaturing solution for 2 x 15 min at room temperature. The gel was rinsed again before being washed, with agitation, in neutralization solution, 2 x 15 min washes at room temperature. The gel was rinsed in dH_2O.

% Agarose gel	Effective separation range (Kb)
0.3	5-60
0.6	1-20
0.7	0.8 -10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

Table 2.3 Alterations to agarose gel % to resolve DNA of different size. This table outlines the different percentages of agarose content in a resolving gel to separate out DNA of varying ranges in size.

Capillary transfer of DNA to Hybond-N membrane

A sheet of 3MM blotting paper was placed over a piece of glass which was longer and wider than the gel. The ends of the blotting paper draped over the edges of the plate, into a tank filled with 20X SSC; transfer buffer. The agarose gel was trimmed to remove any gel that sits proud of the surface around the wells. The gel was placed face down on the blotting paper. Air bubbles between the gel and 3MM paper removed by rolling with a 10ml pipette. Parafilm was placed around the edge of the gel to prevent evaporation and drying out of the blotting paper. The Hybond-N membrane was rinsed in dH_2O and the 20X SSC and placed carefully on the gel surface, ensuring that there were no bubbles between the membrane and the gel. The membrane was not moved once it had been applied to the surface of the gel. Three pieces of 3MM blotting paper wetted in 20X SSC were placed on top of the Hybond-N membrane. Rolling with a 10ml pipette was used to exclude any air bubbles. A 1cm thick pile of 3MM paper, and a 3cm thick pile of paper towels, cut to the same size as the membrane was placed on top of the wet blotting paper. Next a glass plate was placed on top. The transfer was left to sit for 10 min before a weight (a 500ml

bottle half filled with liquid) was placed on top. The transfer was left overnight. See figure 2.3.

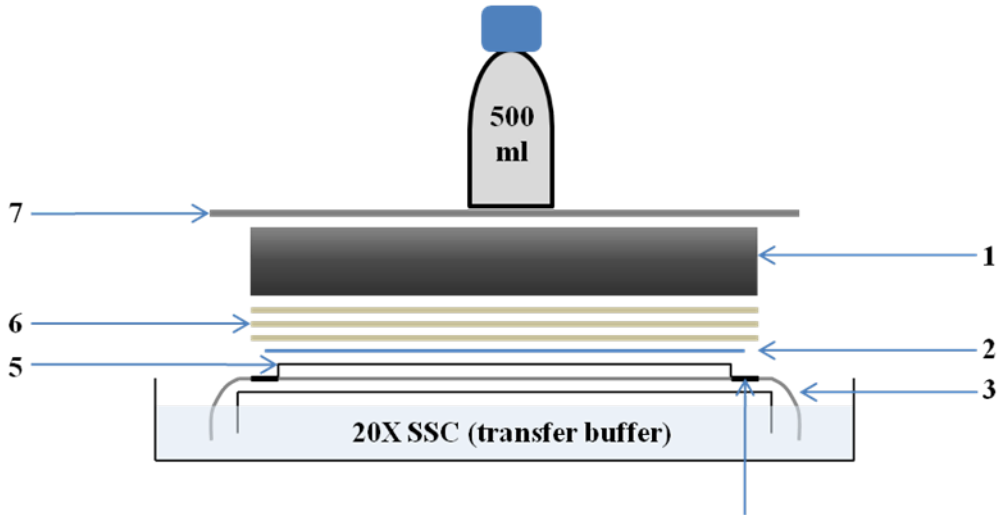


Figure 2.3 Apparatus used for the capillary transfer of DNA to nylon membrane. (1) stack of paper towels (3cm thick), (2) membrane, (3) 3MM blotting paper wick, (4) saran wrap border, (5) agarose gel, (6) stack of 3MM blotting paper (1cm thick), (7) glass plate.

Fixing DNA to membrane

The transfer apparatus was dismantled and the membrane placed DNA side up on a clean sheet of blotting paper. The DNA was fixed to the membrane using the Stratagene UV Stratalinker. (Press: Auto crosslink start).

Southern hybridisation

The membrane was carefully rolled in a nylon mesh and placed in a hybridisation bottle. The membrane was pre-hybridise at 65^0C, in hybridisation buffer with denatured (heated to 95^oC, 5 min; snap cool on ice) ssDNA and tRNA, for 4 – 6 hours.

Labelling and hybridisation of DNA probes

25ng of linear template DNA was placed in a 0.5ml microfuge tube and dH_2O added to a total volume of 11µl. The DNA was denatured by boiling in a PCR block and snap cooling on ice. 4µl of thoroughly mixed High Prime (Roche) and 5µl of 50µCi (α-^{32}P) dCTP was added to the denatured DNA and incubated at 37^oC for 10 min. To stop reaction 2µl of 0.2M EDTA (pH 8.0) was added. To remove unincorporated nucleotides a Sephadex Nick Column (GE healthcare) was used. The liquid was

poured from the column and rinsed once with TE. On a retort stand on a tray behind one of the screens in the hot lab the column was filled with TE which was allowed to run through. The probe was carefully added and allowed to run into column membrane. 400µl of TE was added and the flow through collected as waste. A clean microfuge tube was placed in a rack beneath column and another 400µl TE added. This flow through was collected as probe. To measure incorporation of [^{32}P} dCTP, 2µl of probe was pipetted into liquid scintillant in a plastic scintillation vial and counted using a scintillation counter. A probe of at least 20 – 30 million counts was used. Working behind a Perspex screen the hybridisation buffer was poured from the hybridisation bottle into a 50ml falcon and the denature probe (heated to 95^{o}C, 5 min; snap cool on ice) added to the buffer. Hybridisation buffer and probe were poured back into the hybridisation bottle. The membrane was left to hybridise overnight 65^{0}C, rotating.

Washes

2X SSC, 0.1%SDS wash solution was pre-warm overnight at 55^{0}C.

The contents of the hybridisation bottle were disposed appropriately, the bottle (with membrane still inside) was rinsed with ~ 50ml wash solution. The membrane was washed with 100ml wash solution in the bottle at 65^{o}C for 15 min rotating. The membrane was transferred to a plastic box and washed for 30 min at 65^{0}C with 3 – 4 changes of wash solution. The membrane was removed from the wash, excess solution removed by blotting and wrapped Saran wrap, DNA side down, taking care to avoid creases.

Autoradiography

The membrane was fixed to 3MM blotting paper and two autoradiography rulers (Sigma R8133) placed at different points beside the membrane. The membrane was exposed at –80°C to BIOMAX-MS film for 24 hr and up to 7 days.

2.6.13 Inverse PCR

1µg of genomic DNA containing transposon or control DNA was digested overnight in a 37^{o}C water bath and ligated overnight, at 15^{o}C, to form small circular DNA molecules. The ligation reaction was set up as follows:

Digested DNA (200ng)	4µl
T4 ligase	1µl
10x Buffer	1µl
H_2O	4µl
Final volume	10µl

1st Step PCR was carried out as follow using primers in table 2.4:

DNA – ligation (80ng)	4.0 µl
10x Buffer	2.5 µl
dNTP's 10mMm	0.5 µl
primer 1 (100pmol/µl)	0.1 µl
primer 2 (100pmol/µl)	0.1 µl
Fast start TAQ	0.25µl
H_2O	17.05µl
Final volume	25.0 µl

PCR 94°C/5 min; 94°C/30 s, 50°C/30 s, 72°C/1 min, repeat 35 times; 72°C/5 min

2nd Step PCR was carried out as follow using primers in table 2.4:

DNA – 1st PCR reaction	1.0 µl
10x Buffer	2.5 µl
dNTP's 10mM	0.5 µl
primer 1 (100pmol/µl)	0.1 µl
primer 2 (100pmol/µl)	0.1 µl
Fast start TAQ	0.25µl
H_2O	20.05µl
Final volume	25.0 µl

PCR 94°C/5 min; 94°C/30 s, 50°C/30 s, 72°C/1 min, repeat 35 times; 72°C/5 min

The PCR reaction was resolved on a 1% agarose gel and the PCR product cut from the gel. The DNA gel purified and cloned into pGEM®-Teasy and transformed into DH5 alpha competent cells. Colonies were picked to grow overnight cultures and the DNA retrieved by small-scale plasmid preparation and sent for sequencing.

STEP	PRIMERS: FORWARD (F) REVERSE (R)	ANNEALING TEMPERATURE
1st Step PCR	F: GCAGAAGAGCAGAGAGGATA R: GCGATGACGAGCTTGTTGGC	50°C
2nd Step PCR	F: TCATCGTCTAAAGAACTACC R: GCGCGCCGTCGACATTGATT	50°C
5' integration validation	F: CCAAGACATCAAAATGAGGAGA R: GCGTCAATTTTACGCATGAT	50°C
3' integration validation	F: CACCACTGATTGAAACAGTT R: GTCAATGCGGTAAGTGTCACTG	50°C

PCR conditions: 95°C for 20 minutes; 30 cycles of 95°C for 30s, annealing temperature for 30s and 72°C for I minute, and final extension of 60°C for 30 minutes.

Table 2.4 Inverse PCR primer sequences. Forward and reverse primers are listed for each step in column 1. Annealing temperatures differed between genes and are listed in column 3, the overall cycling conditions did not changed and are outlined below the table.

2.7 CELL CULTURE METHODS

2.7.1 Cryopreservation of cell lines

2.7.1.1 Irradiated and non-irradiated fibroblast cell lines

Cells were washed once with PBS before incubating with trypsin until cells were observed to detach from surface of the culture vessel. Cells were washed in culture medium to inactivate the trypsin and centrifuged at 1000 rpm for 3 min, resuspended in fresh medium (half final freezing volume), and the same volume of freezing medium added to the cell suspension. This suspension was aliquoted into 2ml Nunc cryovials (1.8ml of suspension per tube) and placed in a cryobox and stored at -80°C overnight after which they were transferred to a storage box and stored at -150 °C.

2.7.1.2 Cultured Primordial Germ Cells

Freezing box chilled to 4°C. 1ml of cell suspension placed in 1.5ml screw cap microfuge tube and centrifuged at 1000 rpm for 3 min. The supernatant was removed and the cells resuspended in fresh medium (half total final freezing volume), and the same volume of freezing medium added to the cell suspension. The freezing medium is added drop by drop at approximately 1 drop/s until all has been added. The suspension was mixed gently by pipetting and aliquoted into 2ml Nunc cryovials (1.8ml of suspension per tube) and placed in a cryobox and stored at -80^{o}C overnight after which they were transferred to a storage box and stored at -150 ^{o}C.

2.7.2 Resuscitation of cryopreserved cell lines

2.7.2.1 Irradiated and non-irradiated fibroblast cell lines

Frozen vials of cells were removed from -150^{o}C and placed in a 37^{o}C water bath. After thawing, cells were resuspended in 5ml of medium warmed to 37^{o}C and centrifuged at 1000 rpm for 3 min before plating out in fresh medium.

2.7.2.2 Cultured Primordial Germ Cells

Frozen vials of cells were removed from -150^{o}C and thawed by holding tube firmly in palm of hand or in beaker of room temperature water. Cells were resuspended in 10 ml KO-DMEM in a 15 ml tube. The cell suspension was centrifuged at 1000 rpm for 3min and the supernatant removed. The cells were resuspended in the desired volume of PGC medium and transferred to a culture well pre-seeded with irradiated fibroblast cells (feeders).

2.7.3 Inhibition of signalling pathways in PGCs using small molecule inhibitors

1000 chicken PGCs were deposited in wells on a 48 well tissue culture pre-seeded with STO feeders (1 x 10^5 cells) and filled with 300µl of basic culture medium plus hFGF2 (2ng/ml). Wells were then treated with inhibitor (LY294002, 10µM; PD0325901, 1µM; inhibitor 420099, 1µM) or vehicle (DMSO). Cells were then cultured for seven days under treatment or control conditions. At the seven-day end

point viable cells were counted. Trypan blue dye exclusion was used to distinguish live from dead cells.

2.7.4 Trypan blue staining

Viable cells were counted by dye exclusion with trypan blue. Cell suspension and trypan blue were mixed thoroughly at a ratio of 1:1. 10-20µl of suspension was transferred to a haemocytometer and the viable (unstained) cells were counted.

2.7.5 Transfecting chicken cells with DNA

24 hours prior to transfection plate DF1 cells on a 6 well tissue culture plate so that each well is 60-70% confluent at time of transfection. Before use, allow the vial of FuGENE® HD Transfection Reagent to reach room temperature. Mix by inverting or vortexing briefly. If a precipitate is visible, briefly warm at 37°C, then cool to room temperature. To a sterile microfuge tube add 90–98µl of growth medium prewarmedto room temperature so that the final volume after adding the DNA is 100µl. Add 2µg of each plasmid DNA (0.2–1µg/µl), and vortex. Add 6µl of FuGENE® HD Transfection Reagent directly to medium, and mix immediately. Incubate the FuGENE® HD Transfection Reagent/DNA mixture for 0–15 minutes at room temperature. FuGENE® HD Transfection Reagent/DNA mixture to wells on to return cells to the incubator for 72hrs hours. Measure transfection efficiency using an assay appropriate for the reporter gene. For transient transfection, cells were assayed 72 hours after transfection, for stable transfection 21days after transfection.

2.7.6 Transfecting chicken PGCs with DNA

For each transfection sample, prepare complexes as follows: Aliquot 200 µl of Opti-MEM® I Reduced Serum Medium into a 1.5ml microfuge tube. Mix DMRIE-C before use, then dilute 6 µl into the medium, and mix gently by inversion. In another 1.5ml microfuge tube, dilute 4 µg of DNA in 200 µl of Opti-MEM® I Reduced Serum Medium. Add the diluted DNA to the tube containing diluted DMRIE-C. Mix gently by invering and incubate for 15-45 minutes at room temperature (solution may appear cloudy). While complexes are forming, prepare PGCs, approximately 30,000

chicken PGCs per transfection, by transferring to 200µl Opti-MEM® I Reduced Serum Medium in a well of a 4 well tissue culture plate and added the DNA/DMRIE solution, mix gently by swirling. Incubate cells at 37°C in a CO2 incubator for 4-5 hours. Place transfection in 1.5ml microfuge tube and centrifuge for 3 min at 1000rpm. Resuspend pelleted cells in 300µl of basic culture medium (2.1.6) plus hFGF2 (2.5ng/ml). Test for transgene expression 48-72 hours post-transfection.

2.8 MATHEMATICAL AND STATISTICAL ANALYSIS

2.8.1 Calculating stable transfection

The equation for calculating rates of stable transfection is as follows:

$$\text{Integration Frequency} = \text{\% GFP expressing cells @ d21} \times \left(\frac{100}{\text{\% Transfection Efficiency}} \right)$$

2.8.2 Calculation of standard deviation

Microsoft Excel was used to calculate the standard deviation between the averages for each data set displayed on graphs.

2.8.3 Calculation of standard error of the mean

Microsoft Excel was used to create graphs and calculate the standard error of the mean for each data set represented on graphs. Standard error of the mean was used for all error bars.

2.8.4 General linear models and Post-hoc Tukey comparisons

All analyses were carried using R (version 2.13.1 © 2011 The R Foundation for Statistical Computing) and $P<0.05$ was taken to indicate statistical significance. The analysis was carried as follows:

Figures 3.3 and 3.9

General linear models with binomial errors (GLMb) was carried out to identify overall differences In the percentage of successful isolations between groups. This was followed by standard post-hoc pairwise Tukey comparisons to identify where any differences lay.

Figure 5.3
Analysis of variance with poisson errors was carried out to identify differences in transient transfection rates in chicken DF-1 cells. This was followed by post-hoc pairwise Tukey comparisons of the transfection efficiencies in transposon vectors in the presence of transposase data only.

Figures 5.4 and 5.5
Two-way GLMb was carried out to identify overall differences in stable transfection efficiencies. This was followed by post-hoc pairwise Tukey comparisons of the transfection efficiencies in transposon vectors in the presence of transposase data only.

2.9 COMPUTER PACKAGES AND ONLINE RESOURCES

BLAST searches carried out and gene sequences obtained using:
http://genome.ucsc.edu/index.html

DNASTAR Lasergene 9 core suite for alignment and plasmid maps.

CHAPTER 3: THE CULTURE AND GERM LINE COMPETENCY OF CHICKEN PRIMORDIAL GERM CELLS

3.1 INTRODUCTION

The *in vitro* culture of chicken PGCs has significant potential to study the role of various pathways in germ cell development and as a cell based system for the production of genetically modified chickens. The culture of chicken PGCs *in vitro* has proved challenging despite the cells having been well described and characterised. Manipulation of chicken PGCs for the purposes of producing transgenic poultry offers a relatively straightforward method for the transmission of induced genetic modification to the next generation. As discussed in section 1.5 transgenic chickens have been produced with varying degrees of success by a variety of different methods. It has been demonstrated by several laboratories that chicken PGCs isolated from the circulating embryonic blood or the extra-embryonic germinal crescent when injected into host embryos will undergo differentiation and meiosis as normal, forming functional gametes evidenced by the production of offspring with donor PGC phenotype (Wentworth *et al*. 1989; Vick *et al*. 1993; Vick *et al*. 1993; Petitte *et al*. 1991). It is therefore important to be able to use published work to develop a robust culture system. This chapter aims to repeat and advance the work outlined by Van de Lavoir *et al*. (2006) by demonstrating that chicken PGCs can be isolated from embryonic blood and maintained in culture for more than two months.

3.1.1 The Van de Lavoir PGC culture method

3.1.1.1 The culture method

As outlined in section 1.5.3 a method for the propagation of chicken PGCs has been established (van de Lavoir *et al*. 2006). In this method PGC cultures were established using one to five microlitres of blood collected from Barred Plymouth Rock (BPR) embryos, that had been incubated to between stage 14 and 17 HH, and placed in single wells of a 96 or 48 well tissue culture plate. Tissue culture plates were pre-seeded with STO ($3x10^4$ cells/cm^{-2}) or BRL cells ($1x10^5$ cells/cm^{-2}) that were mitotically inactivated. STO and BRL feeder cells express IGF, LIF and SCF

(Williams *et al*. 1988; Smith, Heath, *et al*. 1988). As STO cells are a mouse line and BRL a rat cell line this may result in differing effects from the secreted cytokines. In the van de Lavoir method no preference for a particular feeder cell is stated and the efficiency based on lines established using either cell type is not defined. The PGCs were isolated from blood cells by culturing in a medium of (KO)-DMEM conditioned on BRL cells (Lanza *et al*. 2004). BRL conditioned medium will be variable between batches within a lab given the process by which it is produced and therefore even more variable between labs. The use of BRL-conditioned medium is a source of much variability when trying to recapitulate a defined protocol. The van de Lavoir method outlines the addition of 6ng/ml SCF and 4ng/ml hFGF2 to the culture medium. Addition of growth factors to this culture medium and that of other cells lines and the requirement for such growth factors *in vivo* has been discussed in chapter 1. In the van de Lavoir method the supplier for both the SCF and hFGF2 is omitted from the outlined protocol, as is the species of SCF used. It was predicted that this could result in difficulties when attempting to recapitulate a protocol due differences in growth factor quality that will exist between suppliers. The lack of species specification for the SCF makes it impossible to accurately repeat the van de Lavoir methodology. However as the amount of supplemented SCF was specified it was assumed that the SCF was commercially obtained product and therefore not chicken SCF (cSCF). Given that SCF is also not functionally conserved between all animals (chapter 4) could also prove problematic when trying to isolate PGCs using the van de Lavoir method. As outlined in the introduction several other components are added to the culture medium 7.5% FCS (supplier not specified), 2.5% CS, 2mM L-glutamine, 1mM sodium (Na) pyruvate, 1x nucleosides, 1x non-essential amino acids and 0.1mM β-mercaptoethanol. Of the 114 individual blood samples taken, 10 male and 2 female cell lines were isolated and maintained in culture. The isolated cells were morphologically round in shape and non-adherent. RT-PCR analysis of cells cultured for 32 to 197 days showed continued expression of the germ-line specific *CVH* (chicken *vasa* homolog) and *DAZL* (Deleted in azoospermia-like) genes. FACS analysis of cells that had been maintained in culture for 280 days also

showed PGC specific expression of CVH. These analyses were used to confirm that the cultured cells were PGCs.

3.1.1.2 Demonstration of germ line transmission

Cells ranging from twenty-nine to 110 days in culture were demonstrated to have retained the ability to form functional gametes (Figure 1.6). 1200 to 3000 cells from each cell line were injected into individual White Leghorn (WL) embryos at stage 13-15 HH of development and the embryos incubated to hatch. Twenty-eight male and fifteen female putative chimeras were crossed to wildtype WLs, all transmitted the BPR phenotype to the next generation at transmission rates ranging from 0.1% to 86%, this was comparable with the transmission rates observed when freshly isolated chicken PGCs are used to make germ line chimeras (Naito *et al*. 1999). Whilst male PGCs could be maintained *in vitro* for 280 days the two female cell lines, PGC56 and PGC85, could not be maintained beyond 109 and 77 days respectively.

3.1.1.3 Addition of cytokines and growth factors

The culture conditions outlined in the published method require a number of components, which by their nature cannot be standardised. This includes sera from chicken and bovine and medium conditioned on Buffalo Rat Liver (BRL) cells. The addition of two exogenous growth factors, FGF2 and SCF are also listed as essential. All these components had to be considered in attempts to recapitulate the published method. Another key cytokine, which was not included in the published method, is SDF1 (CXCL12). SDF1 interaction with its receptor CXCR4, has been associated with a role in PGC migration and survival in mouse and zebrafish (Ara *et al*. 2003; Molyneaux 2003; Doitsidou *et al*. 2002). There is evidence to suggest that SDF1/CXCR4 interaction plays a role in chicken PGC migration (Weidinger *et al*. 2003; Stebler *et al*. 2004) and therefore may promote PGC survival *in vitro*.

3.1.2 Analysis of PGC expression of pluripotency genes

Although propagation of mouse PGCs *in vitro* for more than just a few days has not been achieved, gene profiling *in vivo* has been carried out (Durcova-Hills *et al*.

2008). When mouse PGCs are isolated *in vitro* they can be dedifferentiated into EG cells by the presence of FGF2 or high levels of AKT signalling, which is activated by SCF (Durcova-Hills *et al*. 2006; Kimura *et al*. 2008). EG cells are an ES-like cell and in mouse have been shown to express the same pluripotency genes as ES cells. EG cells have also been shown to contribute to the somatic tissues in the same way as ES cells. Analysis of PGC gene expression in mouse embryos at four time points showed that mouse PGCs express the pluripotency markers *Oct4*, *Nanog* and *Sox2*. Mouse PGCs dedifferentiation to form EG cells coincides with initiation of expression of two other pluripotency genes, *c-Myc* and *Klf4* (Durcova-Hills *et al*. 2008). Pluripotent mouse ES-cells express all five of these genes suggesting that it may be the lack of *c-Myc* and *Klf4* expression that restricts PGCs to the germ cell lineage.

3.2 AIMS

1. Demonstrate that chicken PGCs can be isolated from embryonic blood and propagated for more than 2 months *in vitro*.
2. Demonstrate that after prolonged culture *in vitro* chicken PGCs maintain the ability to colonise the developing gonad.
3. Demonstrate that chicken PGCs cultured *in vitro* can give rise to functional sperm.
4. Characterise cultured PGCs by investigating the expression of germ cell specific and pluripotency genes.

3.3 RESULTS

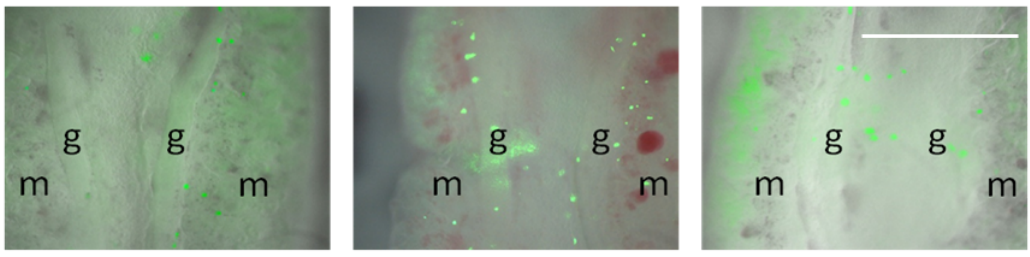
3.3.1 Assessment of embryonic blood for the presence of circulating PGCS

Before attempting the isolation of chicken PGCs, embryonic blood was assessed for the presence of circulating PGCs. Blood was aspirated from the vasculature of five, stage 16 HH GFP^+ ISA brown embryos and injected directly into the vasculature of stage matched, wild-type ISA brown embryos *in ovo*. Donor and recipient embryos were accessed by windowing and after injection the recipient embryos resealed (materials and methods 2.5.4). Recipient embryos were incubated until embryonic day five at which point surviving embryos were sacrificed and their gonads examined. All surviving embryos had GFP^+ cells in the developing gonad although some GFP^+ cells were observed in other intra-embryonic sites, including the mesonepherous (n=3 of 3)(Figure 3.1). This validated that every embryonic blood sample used to initiate chicken PGC cultures had the potential to form a PGC line.

a.

Embryo Injected	GFP expressing cells in gonad
1	+
2	+
3	+
4	n/a
5	n/a

b.



g= gonadal streak m= mesonephros

Figure 3.1 Circulating PGCs in embryonic blood samples. (a) Table listing wild type embryos with GFP^+ cells in the gonadal streak after being injected with embryonic blood aspirated from GFP^+ expressing embryos. (b) The three injected embryos that survived to embryonic day five all had GFP^+ cells in the forming gonad and surrounding mesonephros. Bar 50mm.

3.3.2 Isolation of PGCs using the van de Lavoir method

Fertile eggs from ISA brown chicken hens were incubated at 37^{o}C for approximately 50 hours by which time the majority of embryos had reached stage 16 HH of development. Blood samples of 2-4µl were aspirated from 94 individual embryos and placed in wells of a 48 well plate on feeder cells in medium containing BRL conditioned medium, 6ng/ml SCF and 4ng/ml 7.5% FBS, 2.5% CS, 2mM L-glutamine, 1mM sodium (Na) pyruvate, 1x nucleosides, 1x non-essential amino acids, 0.1mM β-mercaptoethanol, penicillin and streptomycin (Materials and methods 2.1.6). Mouse SCF (mSCF) was added, as the species was not specified in the van de Lavoir method. After two weeks in culture PGCs were identified by their rounded morphology, granulated cytoplasm and lack of adherence to the feeder layer (Figure 3.2).

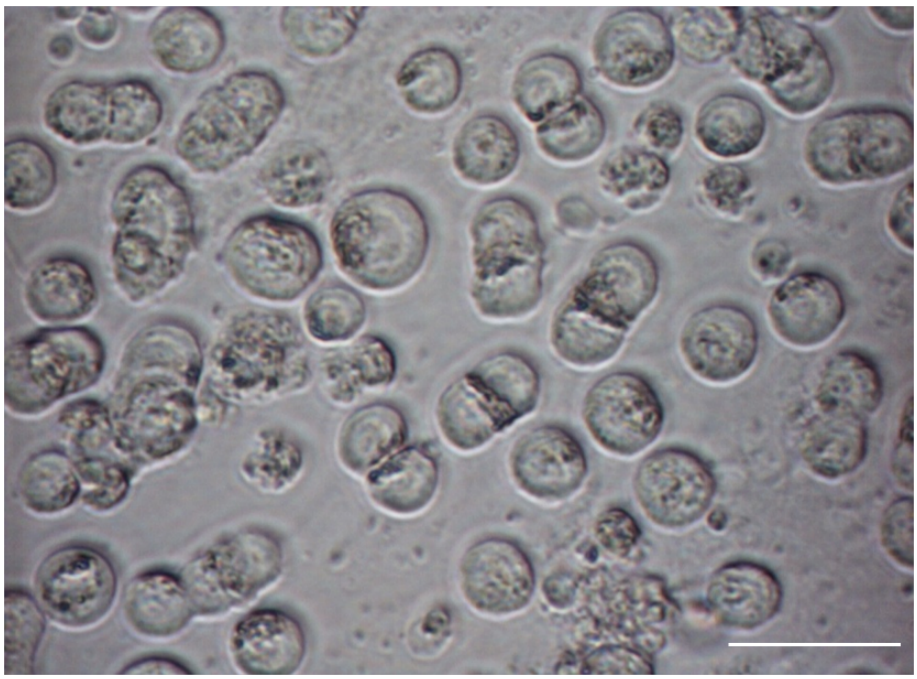


Figure 3.2 Morphology of chicken PGCs *in vitro*. Image showing the characteristic morphology of chicken PGCs in culture. The cells are rounded or oval in shape, have a granulated cytoplasm, often forming doublets and in suspension. Bar 50µm.

Three weeks post aspiration of blood culture wells with 100 or more PGCs present was determined to be successful isolations (Table 3.1). None of the 94 cultures initiated using this method was determined to be successful isolations.

INITIATION DATE	NO. CULTURES INITIATED	NO. WELLS >100 CHICKEN PGCS
26/10/2007	19	0
01/11/2007	20	0
09/11/2007	10	0
15/11/2007	20	0
16/11/2007	15	0
22/11/2007	10	0

Table 3.1 Attempted culture of PGCs using the van de Lavoir method. The number of PGC cultures initiated using the van de Lavoir culture method and the number of cultures that resulted in successful isolation (>100 cells) of PGCs from embryonic blood three weeks post initiation of the culture.

3.3.3 Assessment of sera and growth factors on the isolation of PGCs

Recapitulation of the van de Lavoir method was difficult and did not achieve the desired results of establishing chicken PGC cultures. To address these difficulties the culture medium was investigated, focusing on the effects of sera and growth factors on the propagation of chicken PGCs *in vitro*.

3.3.3.1 Assessment of FBS and chicken sera

The composition of sera is varied and can differ widely from batch to batch. Unknown levels of growth factors and cytokines in sera can exert inhibitory or stimulatory effects on cells that may or may not be desired. For these reasons several commercially available FBS (foetal bovine sera) and chicken sera were tested to determine which of the sera support the propagation of PGCs in culture. Sera were assessed for their ability to support PGC propagation over three weeks. Using the van de Lavoir method media prepared using different FBS and chicken sera, Hyclone defined and Hyclone characterised FBS, PAA laboratories Gold FBS, PAA laboratories ES cell tested, Biosera chicken serum and Sigma chicken serum, were used to prepare culture wells on a 48 well dish. 2-4µl of blood was aspirated from embryos (n=240) and deposited into the different sera conditions. After seven,

fourteen and twenty-one days the cultures were assessed for the presence of chicken PGCs. The sera that supported growth of PGCs in the largest numbers at the end of three weeks (Table 3.2) were, PAA laboratories ES cell tested FBS and chicken serum from either Biosera or Sigma. No initiated cultures were classed as successful isolations.

FBS	TOTAL CULTURES INITIATED	NO. WELLS >100 CHICKEN PGCS
Hyclone Characterised	50	-
Hyclone Defined	30	-
PAA Gold	20	-
PAA ES cell tested	40	-
CHICK SERA	**TOTAL CULTURES INITIATED**	**NO. WELLS >100 CHICKEN PGCS**
Gibco	20	0
Sigma	40	7
Biosera	40	3

Table 3.2 Assessment of sera on the isolation of chicken PGCs from embryonic blood. Foetal bovine sera (FBS) and chicken sera were assessed for ability to support PGC isolation from embryonic blood. Total cultures initiated refers to the number of blood samples taken to isolated PGCs. Successful PGC isolations refers to initiated cultures with more than 100 PGCs in the well three weeks post culture initiation.

3.3.3.2 The effects of hFGF2 and mSCF on PGC isolation

Using the selected FBS and chicken serum a series of experiments to determine the growth factors were necessary for chicken PGC propagation *in vitro* were carried out. Comparative experiments were set up using 2-4µl of aspirated blood split between two wells on a 48 well dish. On average twenty wells were set up per experiment, ten with one culture condition and ten with another. Four different conditions were assessed: 1. Basic medium (Materials and methods 2.1.6), equivalent to the van de Lavoir medium without growth factors added (No growth factors), 2. Basic medium with 50ng/ml hFGF2 added (FGF only), 3. Basic medium with 6ng/ml mSCF added (SCF only) and 4. Basic medium with 4ng/ml hFGF2 and 6ng/ml mSCF added (FGF+SCF), equivalent to the van de Lavoir media. As described previously cultures were observed at seven-day intervals and only wells containing

100 or more PGCs at three weeks post aspiration of blood determined to be successful isolations (Table 3.3).

Added growth factors	Total cultures initiated	No. wells >100 chicken PGCSs 3 weeks	Successful isolations(%)
No growth factors	130	13	10
hFGF2 4ng/ml + mSCF 6ng/ml	80	10	12.5
hFGF2 4ng/ml	110	25	22.7
mSCF 6ng/ml	50	1	2
hFGF2 2ng/ml	100	31	31
hFGF2 1ng/ml	20	6	30
hFGF2 4ng/ml + mSCF 6ng/ml + SDF1	30	5	16.7
hFGF2 4ng/ml + SDF1	20	6	30
mSCF 6ng/ml +SDF1	10	0	0
SDF1	30	1	3.3
1/2 SDF1	10	3	30

Table 3.3 Identification growth factors added to medium that support chicken PGC isolation *in vitro*. The table lists attempted and successful isolation of chicken PGCs from embryonic blood under different culture conditions. Blood was isolated from ISA brown chicken embryos and deposited in a basic culture (materials and methods) to which different growth factors were added. 590 cultures were initiated from 295 individual embryos and 11 different culture conditions assessed for ability to support PGC survival *in vitro* for three weeks. Successful isolations are cultures with 100 or more PGCs three weeks post initiation.

Cultures (n=370) were initiated, from 185 individual embryos, using the four conditions (Table 3.3). All four conditions resulted in successful isolation of 100 or more PGC after three weeks of culture but at varying efficiencies (Figure 3.3). Under culture condition 1, no growth factors (n=130), isolation of chicken PGCs was observed in 10% of the cultures initiated, this was comparable to 12.5% under condition 4. FGF+SCF. When cultures were initiated using culture condition 3, SCF only (mSCF, n=50), only 2% of cultures resulted in the isolation of 100 or more PGCs at the three week time point this was lower than observed in either condition 1 or 4. In contrast, PGCs were isolated from 22.7% of the cultures initiated in the FGF only medium (culture condition 2, n=110). Standard posthoc pairwise tukey

comparison of these four culture conditions revealed that using SCF only or no growth factor conditions was significantly less ($p<0.043$) effective at deriving PGC cultures than the FGF only medium.

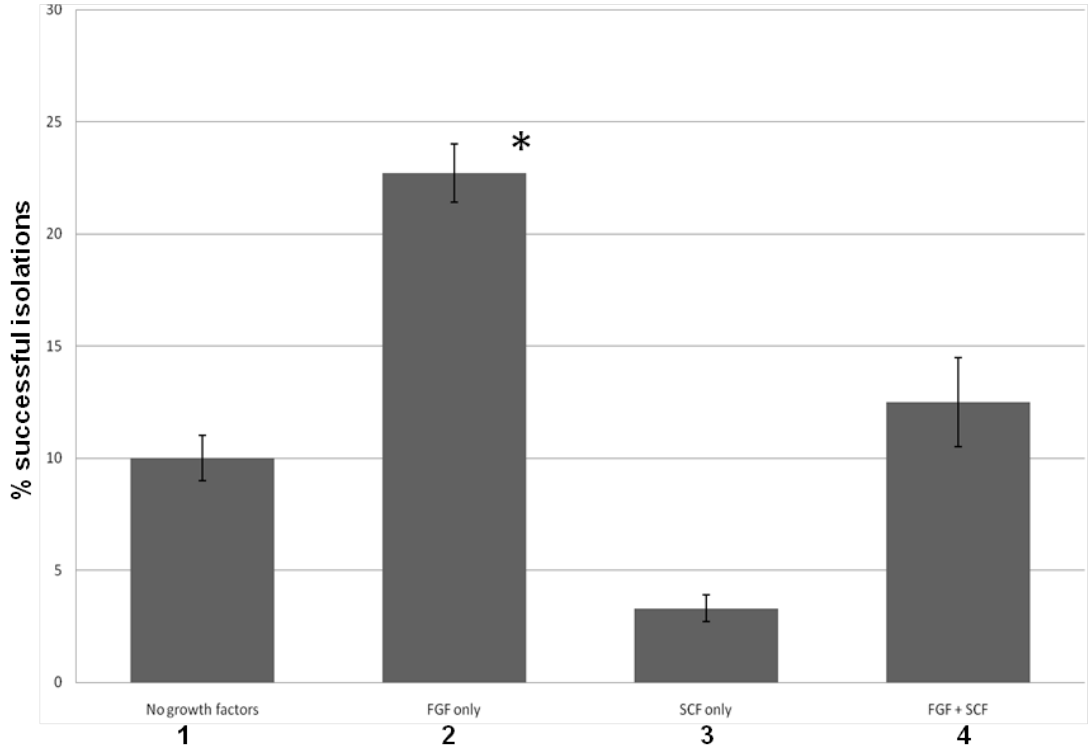


Figure 3.3 Isolation of chicken PGCs from embryonic blood. Graph showing the percentage of successful chicken PGC isolations from all initiated cultures. Cultures were initiated by seeding culture wells with embryonic blood. Embyonic blood was deposited in culture medium with and without growth factors, hFGF2 and mSCF. Four conditions were evaluated: **1.** basic culture medium (Materials and methods, No growth factors), basic cultures medium plus **2.** hFGF2 (FGF only); **3.** mSCF (SCF only) and **4.** hFGF2 and mSCF

3.3.4 Propagation of chicken PGC lines *in vitro*

A total of seven PGC lines were successfully established, six using the FGF only medium (Table 3.4). Four PGC lines; PGC1, PGC23, 3.08.09, 08.08.09 and 03.10.10 were derived from wildtype chicken embryos whilst another two, 10.08.09 and 06.10.10, were both isolated from GFP expressing embryos. All seven of the PGCs lines were identified to be PGCs based on their morphology (Figure 3.4) and were maintained in culture for at least two months. A total of four lines were cryopreserved and could be resuscitated successfully from frozen.

PGC Line	Derivation date	GFP expressing	Cryopreserved
PGC 1	08/08/2008	N	N
PGC 23	08/08/2008	N	N
03.08.09	20/03/2009	N	Y
08.08.09	20/03/2009	N	Y
10.08.09	20/03/2009	Y	N
03.10.10	30/04/2010	N	Y
06.10.10	30/04/2010	Y	Y

Table 3.4 Established chicken PGC lines. This table shows seven chicken PGC lines that were established from embryonic blood samples. All seven lines were propagated in culture for at least two months. Lines were established from five wild type and two GFP^+ embryos. Four of the lines were successfully cryopreserved and could be resuscitated.

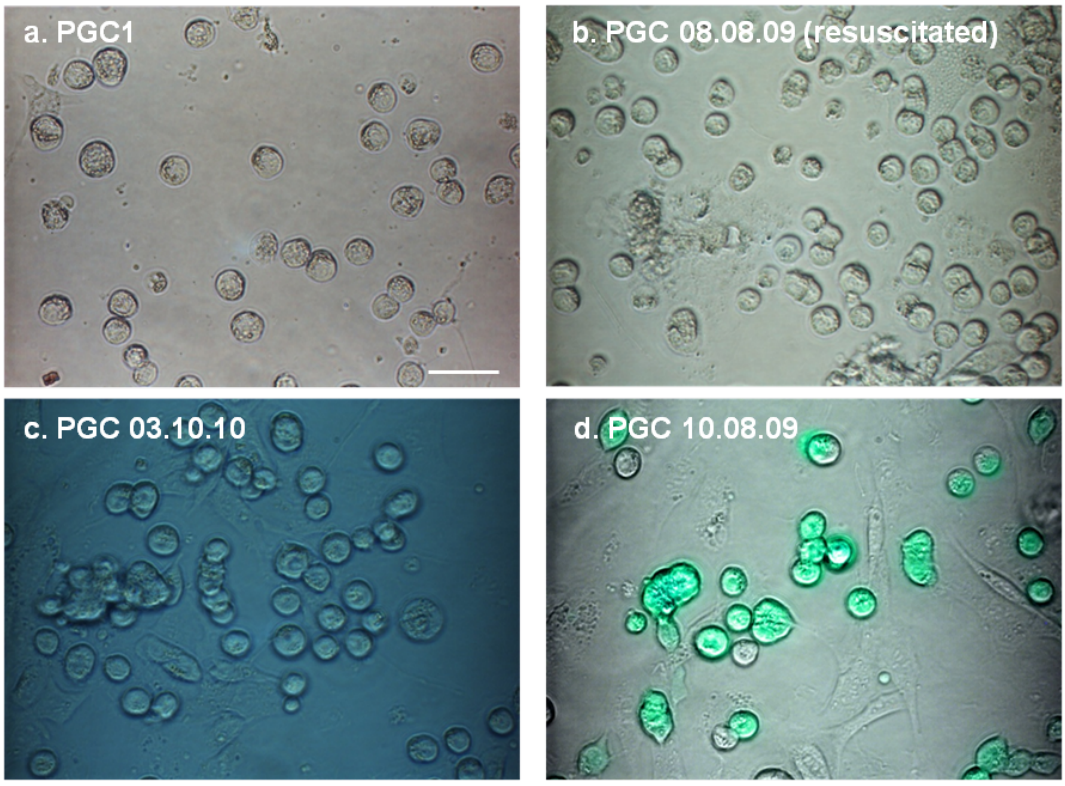


Figure 3.4 Established chicken PGC lines. Images of four of the chicken PGC lines that were established from individual wild type embryos; (a) PGC 1, (b) PGC 08.08.09, (c) PGC 03.10.10 and GFP^+ embryos; (d) PGC 10.08.09. Bar 50µm.

All seven PGC lines were screened and identified to be male by the absence of the Xho repeat on the W-chromosome. This was determined using RNA from each line as a template and Xho specific primers. RT-PCR analysis carried out by L. Taylor (primers listed in Table 2.2).

During extended *in vitro* culture adherent cells were seen to spontaneously form and looked very similar to fibroblasts. It was determined that these cells were chicken PGC derived as they were easily observed in the GFP^+ PGC cultures by their expression of GFP (Figure 3.5). There is some evidence to suggest that some chicken PGCs within cultures will spontaneously dedifferentiate into EG cells and that this can be promoted by the removal of hFGF2, SCF and serum (van de Lavoir *et al*. 2006). Prolonged exposure to FGF2 results in dedifferentiation to EG cells in mouse cultures (Kimura *et al*. 2008) and all seven of the PGC lines that were established were maintained in medium with hFGF2 added.

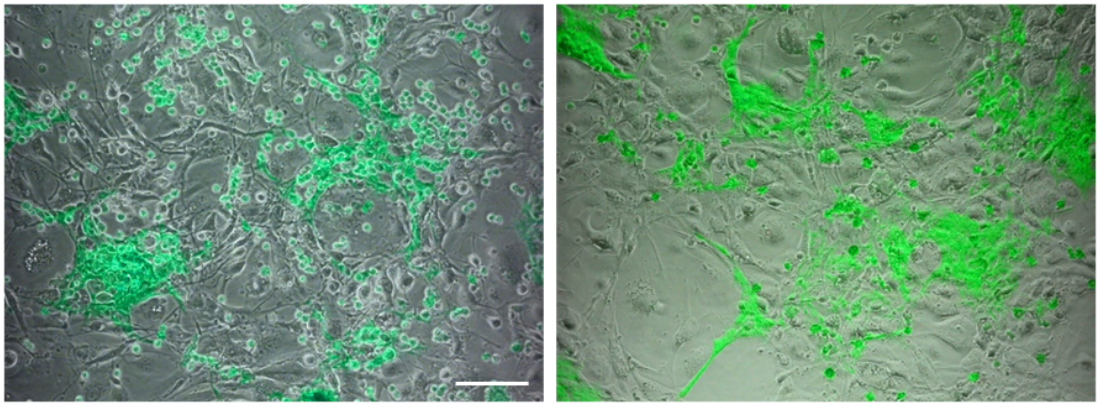


Figure 3.5 Chicken PGC derived adherent cells. Adherent cells formed spontaneously in some of the GFP^+ chicken PGC culture. These cells were GFP^+ confirming that they were derived from the chicken PGCs. Bar 200µm.

3.3.5 The effect of SDF1 on the isolation and propagation of PGCs

3.3.5.1 Assessment of SDF1 receptor expression by chicken PGC lines

SDF1 may play a role in the migration and survival of PGCs in chickens (Nakamura *et al*. 2003). To examine if SDF1 has a role in the proliferation of chicken PGCs *in*

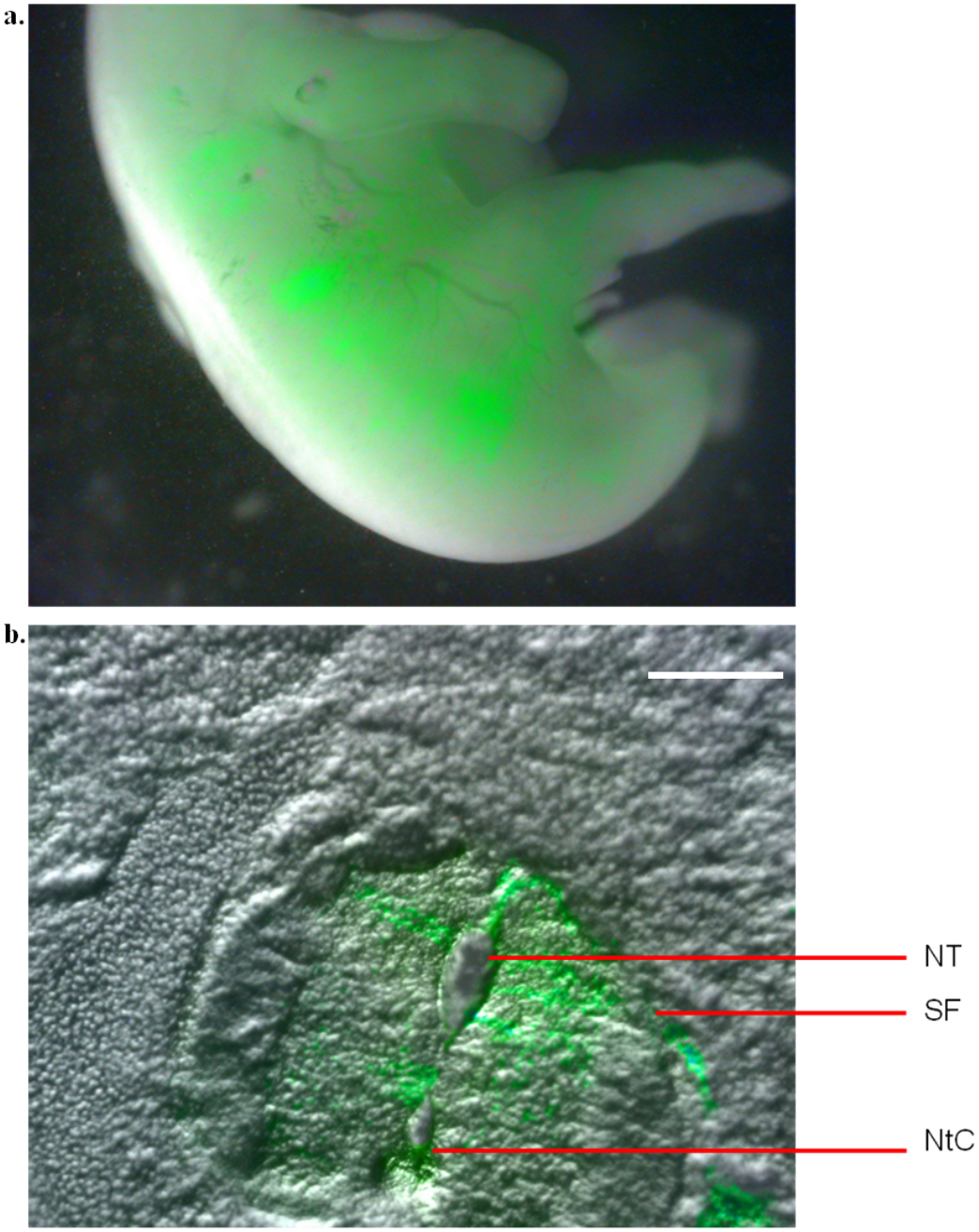


Figure 3.6 GFP+ ES cell contribution in the developing embryo. Chicken ES cells were established from blastodermal cells isolated from the area pellucida of a GFP^+ stage X embryo (EG&K). The cells were assessed for their ability to contribute to the somatic tissues by injection into the subgerminal cavity of a wildtype stage X embryos which were incubated to embryonic day 10. (a) GFP^+ cells are present throughout the body of the embryo and (b) within in the neural tube, notochord and developing limb. Bar 200µm.

vitro expression of the SDF1 binding receptors, CXCR4 and CXCR7 was assessed first. RNA was extracted from an established PGC line PGC08-08-09, nine-day embryonic gonad, a chicken embryonic fibroblast (CEF) line and chicken ES (cES) cells. The nine-day embryonic gonad tissue was isolated from male and female embryos, CEF cells were established from the carcases of nine-day embryos with the viscera removed and then isolated for RNA extraction. The cES cell cultures were set up from the pluripotent blastodisc cells isolated from the stage X HH embryo, the cultures were established by A. Braun. To demonstrate that these cells contributed to the somatic tissue the cES cells were removed from culture suspended in KO-DMEM and using a microcapilliary tube were injected into the subgerminal cavity beneath the blastoderm of a stage X HH embryo. The injected embryos were cultured to hatch using the surrogate shell method (Perry, 1988). Three embryos survived to embryonic day 8. GFP+ cells were identified in two of the embryos (Figure 3.6a). Transverse section of one of the embryos showed that GFP^+ cells were present in the neural tube and the forming limb (Figure 3.6b). The RNA was then used as a template to use CXCR4 and CXCR7 specific primers (Table 2.1) to screen for the presence of the receptor mRNAs (Figure 3.6a and b).

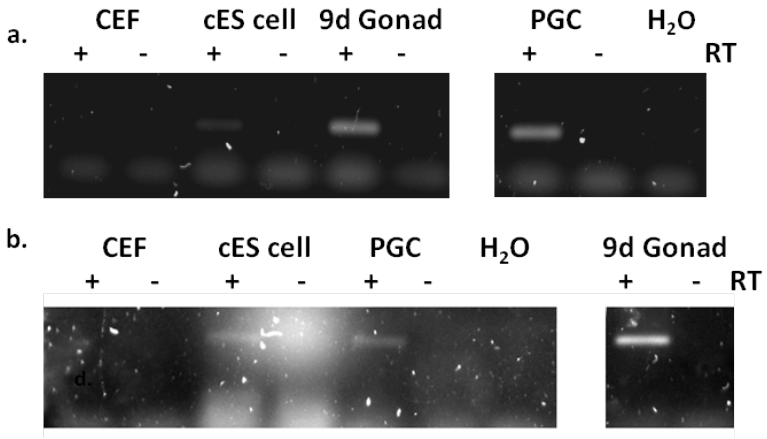


Figure 3.7 Cultured chicken PGCs express receptors for SDF1. RT-PCR analysis was carried out to identify that cultured chicken PGCs expressed the SDF1 receptors (a) CXCR4 and (b) CXCR7. RNA from chicken embryonic fibroblasts (CEF), chicken ES (cES) cells, 9 day embryonic gonad (9 day gonad) and one PGC line was used as a template for receptor specific primers. Expression was observed in the cES cells, the 9 day gonad and the PGCs for both receptors. The CEFs did not express either receptor.

The results in figure 3.7a and b show that the chicken PGC line, cES cells and the nine-day embryonic gonad sample all expressed both CXCR4 and CXCR7. Expression of CXCR4 and CXCR7 in the nine-day embryonic gonad may demonstrate that the expression of these genes in the cultured PGCs was indicative of chicken PGCs *in vivo* and not an artefact of the *in vitro* culture system.

3.3.5.2 Assessment of effect of SDF1 on established chicken PGC lines

Having established that the PGCs expressed the receptors for SDF1 binding, the effect of SDF1 in culture was assessed. Chicken PGC line 08.08.09 was used to seed wells on a 48 well tissue culture plate (n=8). Approximately 1000 chicken PGCs were added to each well pre-seeded with STO feeders and 300µl of the basic culture medium plus hFGF2 (4ng/ml). 25ng/ml mouse SDF1 (mSDF1; dissolved in DMSO) was then added to half the wells (treated) whilst the equivalent volume of DMSO was added to the other wells (controls). Cultures were maintained for two weeks, with cell counts carried out at seven and fourteen days (Figure 3.8a). After seven days in culture the number of PGCs in the control and mSDF1 treated wells were comparable. After fourteen days in culture the number of PGCs in the mSDF1 treated wells was significantly higher than the number of PGCs in the control wells ($p<0.02$). The number of PGCs in the wells treated with mSDF1 increased 12 times more rapidly than those grown in the basic culture medium plus hFGF2 (4ng/ml).

3.3.5.3 Establishing if SDF1 is present in the van de Lavoir culture medium

Addition of inhibitor AMD3100 was used to assess if SDF1 was present in van de Lavoir culture medium and that the effect observed on cellular proliferation resulted from interaction with CXCR4. AMD3100 is a CXCR4 antagonist that blocks SDF1/CXCR4 interaction. If SDF1 were present in the basic culture medium plus hFGF2 (4ng/ml), addition of AMD3100 would result in an inhibition in cellular proliferation. Four different culture conditions were used: 1. basic culture medium plus hFGF2 (4ng/ml) (control) and basic culture medium plus hFGF2 (4ng/ml) plus

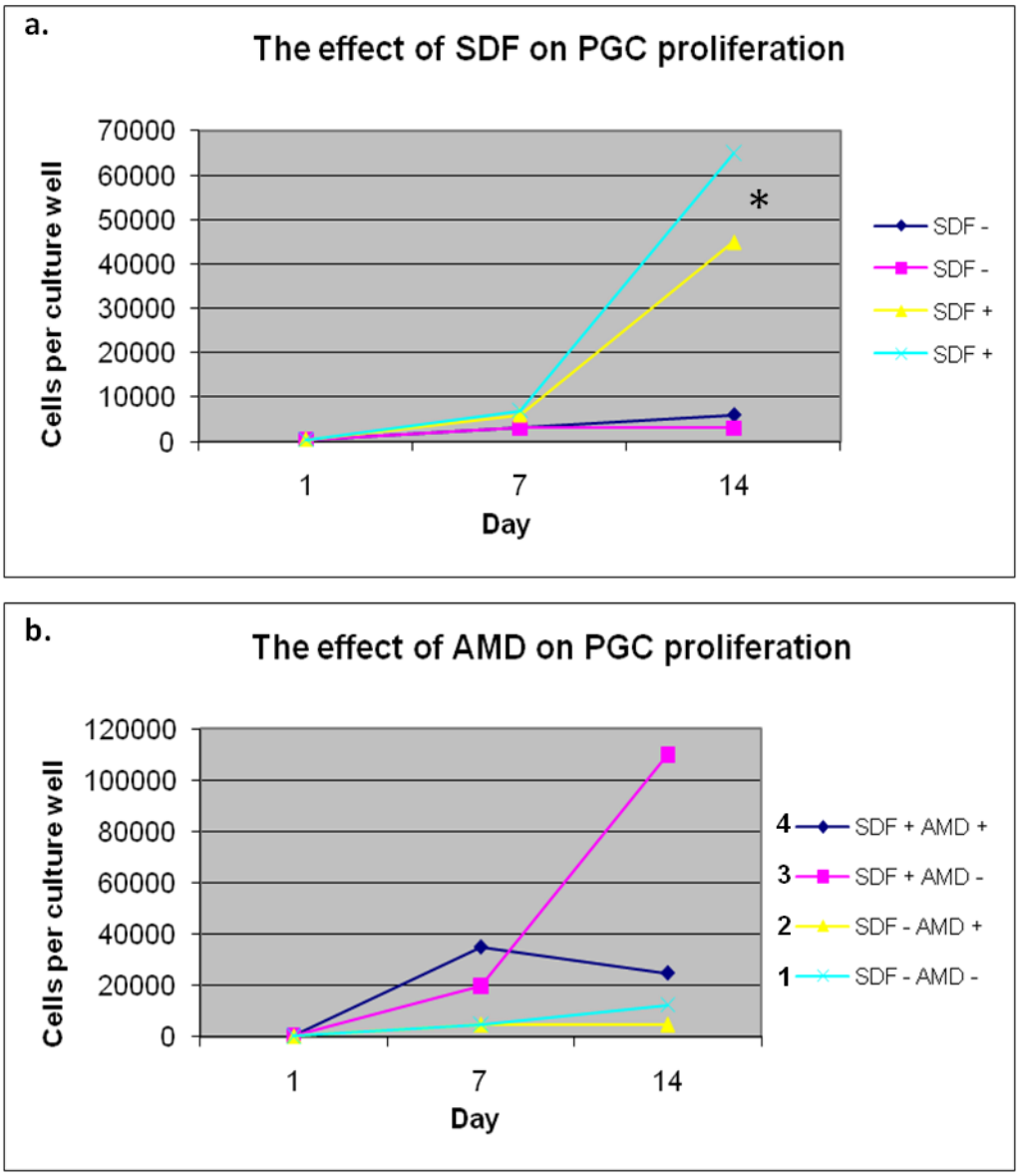


Figure 3.8 The effect of SDF1 on chicken PGC cultures. Established chicken PGC lines were used to determine the effects of SDF1 in the culture medium. (a) Chicken PGCs were culture in presence (SDF+) or absence (SDF-) of SDF1 (25ng/ml). *, $p<0.02$. To determine if SDF1 was present in the basic culture medium (Materials and methods) chicken PGCs were grown in the presence and absence of the SDF1/CXCR4 inhibitor AMD3100. (b) Four different culture conditions were tested, 1. basic culture medium plus hFGF2 (SDF- AMD-), basic culture medium plus hFGF2 and 2. AMD3100 (SDF- AMD+), 3. SDF1 (SDF1+AMD-) and 4. SDF1 and AMD3100 (SDF+AMD+, control for AMD activity). For both (a) and (b) The number of chicken PGCs per culture was 500 at day 1 and were counted at 2 time points over the course of 14 days. These counts were used to generate the growth curve.

2. AMD3100; 3. mSDF1; 4. mSDF1 and AMD3100. mSDF1 was added at (25ng/ml) and AMD3100 at 10µg/ml. Chicken PGC line 08.08.09 was used to seed wells on a 48 well tissue culture plate (n=4). Approximately 1000 PGCs were added to each well pre-seeded with STO feeders and 300µl of one of the four culture mediums at day 0. The number of PGCs in each well was then counted at seven and fourteen days (Figure 3.8b). When the PGCs were culture in basic medium plus hFG2 or basic medium plus hFGF2 and mSDF1 the number of cells at seven and fourteen days was comparable to what was observed in the previous experiment under the same conditions (Figure 3.8a). When the PGCS were grown in basic medium plus hFGF2, mSDF1 and AMD3100 the number of cells at seven days was comparable to cell cultures treated with only mSDF1. After 14 days the effects of the inhibitor could be observed with an obvious decrease PGC number to a total fewer than had been observed at seven days (Figure 3.8b). This was much less than the total number of cells observed in the mSDF1 culture conditions. These results show that AMD3100 is effectively inhibiting SDF1/CXCR4 interaction. When AMD3100 alone was added to basic culture medium plus hFGF2 the increase in PGC number was comparable at seven days to the result observed under the basic culture medium plus hFGF2 conditions. The number of cells in the AMD3100 well did not change between day seven and day fourteen whilst the cell number in the basic culture medium plus hFGF2 conditions had increased, suggesting a steady state between proliferation and apoptosis in the presence of AMD3100. In comparison the 2-fold increase in cell number observed from day seven until day fourteen under the basic culture medium plus hFGF2 (4ng/ml) conditions suggests that AMD3100 may be inhibiting the effects of SDF1 already present in the culture medium. Although this data indicates that SDF1 is already present in the culture medium, source unknown, this experiment would have to be repeated for any statistically validated conclusion to be made.

3.3.5.4 The effect of SDF1 on the isolation of PGCs

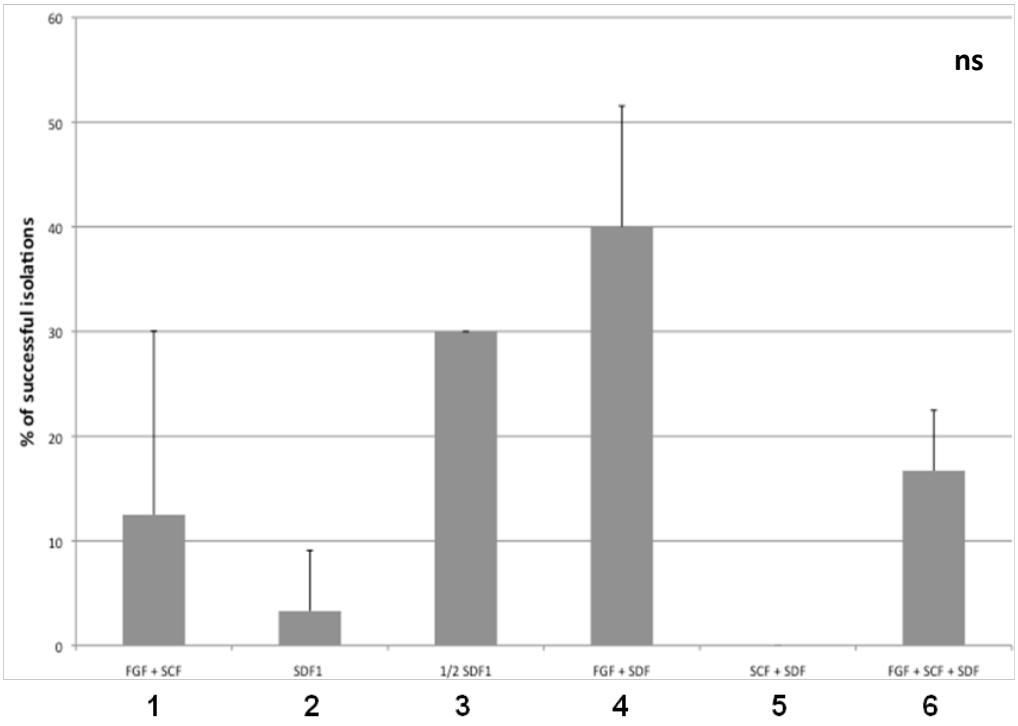
Having demonstrated that mSDF1 in medium promoted expansion of chicken PGC numbers in culture, mSDF1 was assayed for its effects on PGC isolation from

embryonic blood. Comparative experiments were set up using 2-4µl of aspirated blood split between two wells on a 48 well dish. On average twenty wells were set up per experiment, ten with one culture condition and ten with another. Cultures (n=100) were initiated, from blood from 50 individual embryos, across five culture conditions (Table 3.3, Figure 3.9a). The culture conditions tested were basic medium plus 1. mSDF1 (25ng/ml; SDF1); 2. mSDF1 (12.5ng/ml, ½ SDF1); 3. hFGF2 and mSDF1 (4ng/ml and 6ng/ml respectively; FGF+SDF); 4. mSCF and mSDF1 (6ng/ml and 25ng/ml respectively; SCF+SDF) and 5. van de Lavoir medium plus mSDF1 (25ng/ml). These results were compared to the results from using the van de Lavoir medium (FGF and SCF) that were presented in section 3.3.3.2 and figure 3.3 previously. At three weeks post aspiration of blood, cultures with 100 or more PGCs present were determined to be a successful isolation. Only 3.3% of cultures initiated in culture medium 1 resulted in successfully isolated PGCs after three weeks in culture. This was far less than three of the other culture conditions tested, 2 (30%), 3 (40%) and 5 (16.7%) and the van de Lavoir medium (12.5%). Only 10 wells were set up under the conditions of culture medium 4, SCF+SDF, none of these cultures resulted in the successful isolation of PGCs. Standard posthoc pairwise tukey comparison of these culture conditions revealed that no significant increase in derivation efficiency resulted from addition of SDF1 to culture conditions. When cultured in the presence of mSDF1 PGCs formed large floating colonies some of which became adherent and appeared morphologically to be EG cell-like (Figure 3.9b). Due to this morphological change, mSDF1 was not used to maintain cultures *in vitro*.

3.3.6 Evaluation of pluripotency gene expression in cultured PGCs

Initial assessment and determination of chicken PGC cultures has been based on the known cellular characteristics. To determine that these cells were genetically chicken PGCs they were assessed for the expression of marker genes. The genes screened were determined based on the literature for both chicken and mouse (Tsunekawa at al. 2000; Durcova-Hills *et al*. 2008). Differential expression of several genes including some markers of puripotency (oct4, Nanog, c-myc, cSox2 and klf4) which

a.



b.

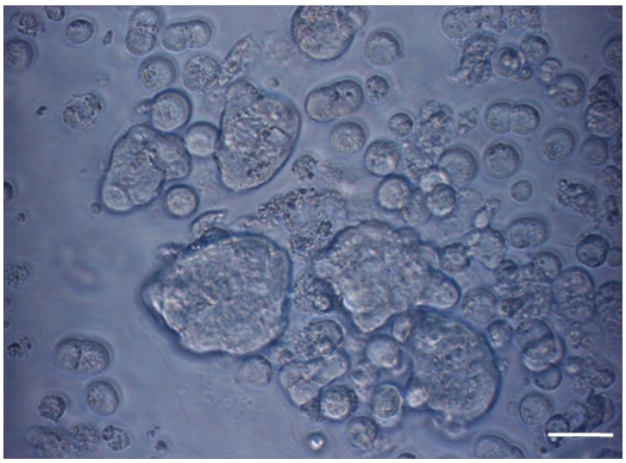


Figure 3.9 The effect of SDF1 supplementation on chicken PGC isolation from embryonic blood. (a) Graph showing the percentage of successful chicken PGC isolations from all initiated cultures. Cultures were initiated by seeding culture wells with embryonic blood. Embryonic blood was deposited in culture medium with and without growth factors, SDF, hFGF2 and mSCF. Six culture conditions were evaluated: **1.** van de Lavoir medium (FGF+SCF)), basic cultures medium (materials and methods) plus **2.** 25ng/ml SDF1 (SDF); **3.** 12.5ng/ml SDF1 (1/2 SDF), **4.** hFGF2 and 25ng SDF1 (FGF+SDF), **5.** mSCF and 25ng/ml SDF1 (SCF+SDF) and **6.** van de Lavoir medium plus 25ng/ml SDF1 (FGF+SCF+SDF). Successful chicken PGC isolations refers to culture wells with more than 100 PGCs present three weeks post initiation. Error bars, SEM. (b) Representative image of chicken PGCs forming floating colonies when grown in the presence of added SDF1. Bar 50µm.

have been described as markers for the dedifferentiation of PGCs to EG cells in mouse were assessed (Durcova-Hills *et al*. 2008). To carry out gene expression analysis, RNAs from eight cell lines and one tissue sample were used as a template. Four cultured chicken PGC lines, 08-08-09, 03-08-09, PGC1 and PGC23 all of which morphologically resembled PGCs were used. One chicken embryonic fibroblasts (CEF) cell line established from day nine embryos as described in section 3.3.5.1 and two chicken ES cell lines (cES1 and cES2), established by M. McGrew confirmed to contribute to the three germ layers (Macdonald *et al*. 2010, supplementary data) using the method outlined in section 3.3.5.1. RNA was also extracted from embryonic 4-day gonad tissue; it was thought that this would provide a suitable positive control sample. Analysis of the STO feeder cell line was also carried out to exclude the possibility of false positives as a result of STO cell contamination in the PGC samples.

All RNA samples assessed for the expression of *CVH, cPouV*, *cNanog*, *cSox2*, *c-Myc* and *cKlf4*. Assessment of GAPDH (chicken) and β-actin (Mouse, STO cells) expression in all samples was used as RT-PCR controls. All chicken derived samples were positive for GAPDH expression (Figure 3.10a). The STO cells were positive for β-actin (Figure 3.10b) but negative for all other genes analysed, confirming that any positive results from the PGC samples were not caused by STO cell contamination. Only the PGC samples were identified to express the germ cell specific marker *cvh* (Figure 3.10c). This confirmed that the cells isolated from embryonic blood were PGCs. Both cES cell samples were positive for expression of all five pluripotency genes, *cPouV*, *cNanog* and *cSox2* (Figure 3.11), *c-Myc* and *cKlf4* (Figure 3.12). This was in keeping with mouse ES cell gene expression. Interestingly all four PGC lines were also positive for the four pluripotency genes. This data is in contrast tomouse PGCs that have been shown not to express *c-Myc* or *Klf4*. However the CEF cells which were shown to be negative for *cPouV*, *cNanog* and *cSox2* (Figure 3.11) were positive for *c-Myc* and *cKlf4* expression (Figure 3.12). Primers and PCR temperatures listed in table 2.2.

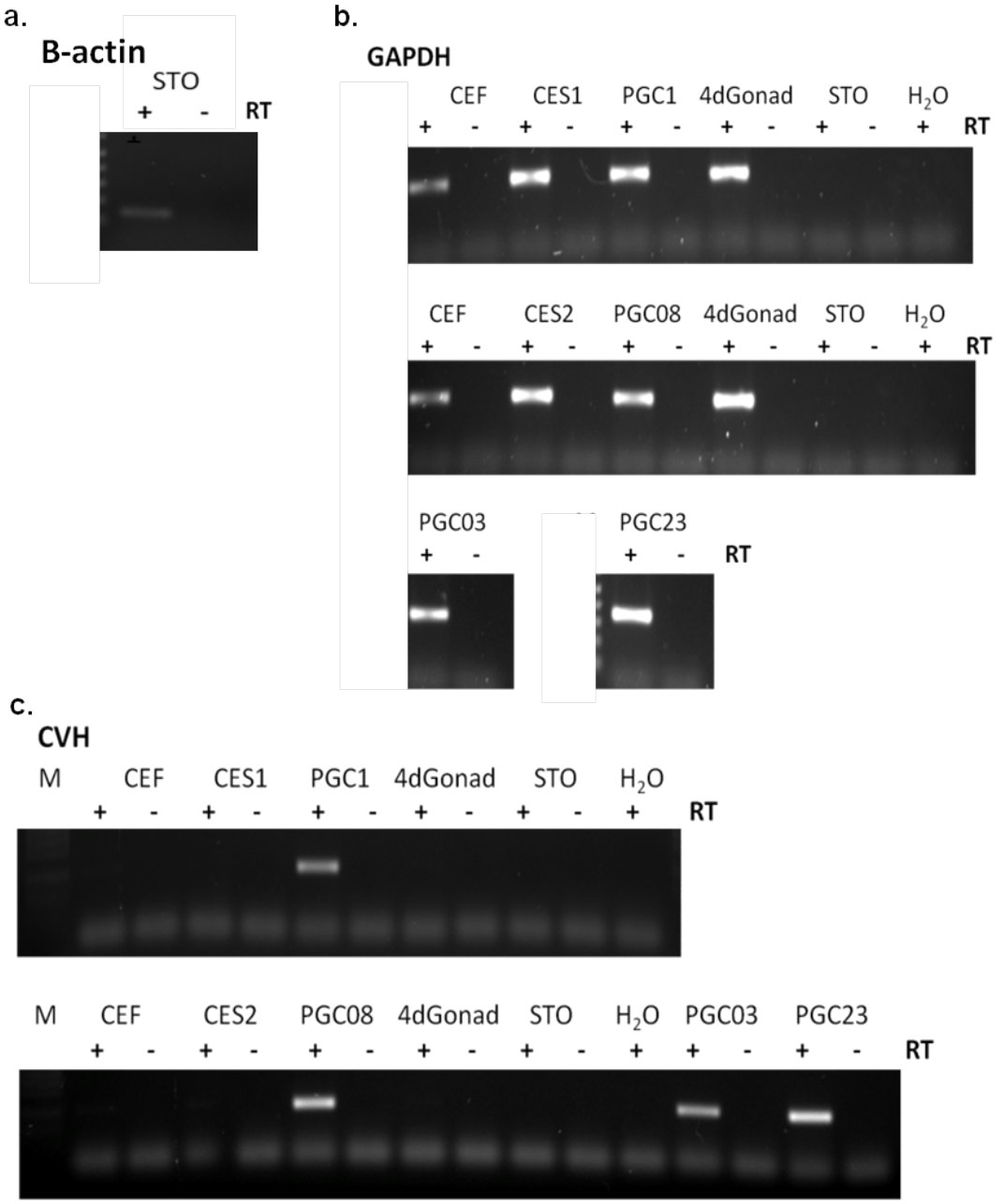


Figure 3.10 CVH expression in chicken cell lines. RNA from chicken embryonic fibroblasts (CEF), chicken ES cells (CES1 and CES2), four chicken PGC lines: PGC1 (PGC1), PGC08.08.09 (PGC08), PGC03.08.09 (PGC03) and PGC23 (PGC23); day 4 embryonic gonads (4d gonad) and mouse feeder cells (STO), were used as a template for analysis of gene expression. (a) GAPDH and (b) β-actin were used as PCR controls for the chicken and mouse samples respectively. Negative controls were performed without reverse transcriptase (RT). (c) CVH expression was identified in only the chicken PGC samples.

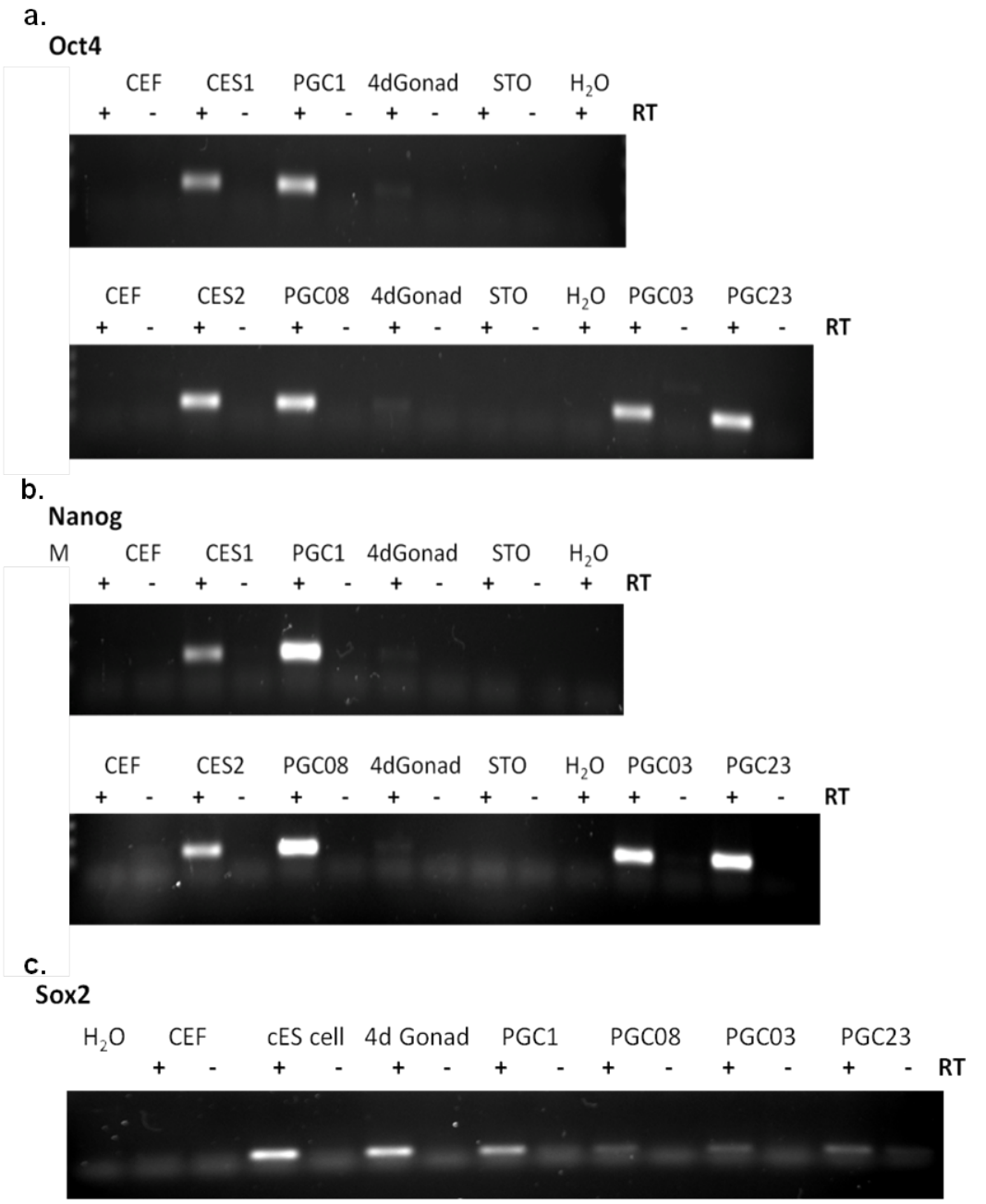


Figure 3.11 Analysis of pluripotency gene expression. RNA from chicken embryonic fibroblasts (CEF), chicken ES cells (CES1 and CES2), four chicken PGC lines: PGC1 (PGC1), PGC08.08.09 (PGC08), PGC03.08.09 (PGC03) and PGC23 (PGC23); day 4 embryonic gonads (4d gonad) and mouse feeder cells (STO), were used as a template for RT-PCR analysis of gene expression. Negative controls were performed without reverse transcriptase (RT). 30 cycles of PCR were used to detect the expression of (a) Oct4 (cPouV), (b) Nanog and (c) Sox2. PCR product was present in the chicken ES cell, chicken PGC and 4 day gonad samples but absent in CEF and STO cells.

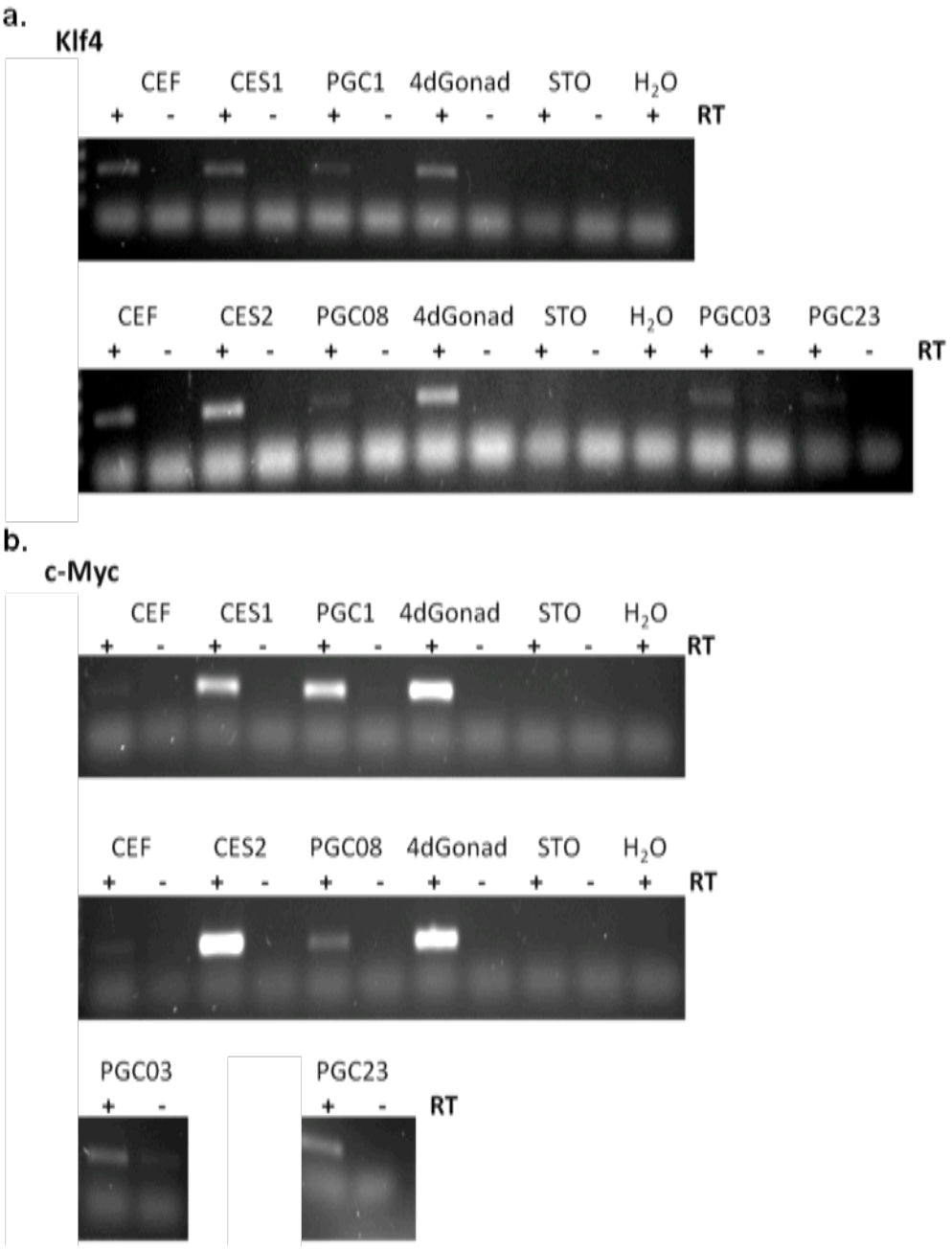
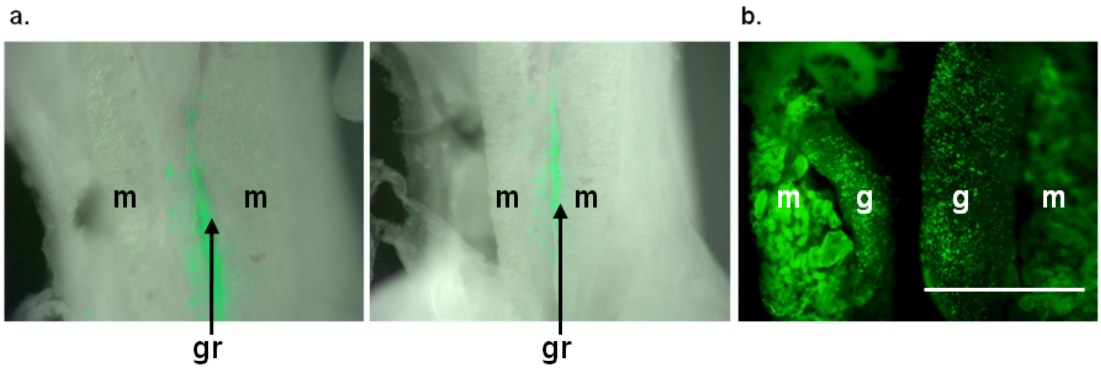


Figure 3.12 Analysis of Klf4 and c-Myc gene expression. RNA from chicken embryonic fibroblasts (CEF), chicken ES cells (CES1 and CES2), four chicken PGC lines: PGC1 (PGC1), PGC08.08.09 (PGC08), PGC03.08.09 (PGC03) and PGC23 (PGC23); day 4 embryonic gonads (4d gonad) and mouse feeder cells (STO), were used as a template for RT-PCR analysis of gene expression. Negative controls were performed without reverse transcriptase (RT). 30 cycles of PCR were used to detect the expression of (a) c-Myc and (b) Klf4 in several cell lines and the embryonic chicken gonad. These genes are expressed in mouse ES and EG cells but not mouse PGCs. PCR product was present in all chicken samples including the four PGC lines.

3.3.7 Cultured chicken PGC colonisation of the developing gonad

3.3.7.1 Assessment of chicken PGC migration in a host embryo

After establishing that the cultured cells were PGCs based on morphology and gene expression they were tested for their ability to migrate *in vivo*. To assess the cells ability to migrate to and colonise the gonad, cells from male chicken PGC lines 10-08-09 and 06-06-08, isolated from GFP^+ transgenic chickens, were injected into host wild type embryos (stage 16 HH) *in ovo*. Approximately 1000 PGCs were injected per embryo. The manipulated embryos were resealed and incubated at 37°C until embryonic day five or day ten. The sacrificed embryos were examined for GFP^+ cells in the developing gonad (n=6). At five days of incubation GFP^+ cells were observed in the ventral midline (Figure 3.13a) and by embryonic day ten were distributed throughout the developing gonad (Figure 3.13b).



gr= genital ridge, g= embryonic gonad , m= mesonephros

Figure 3.13 GFP^+ chicken PGC colonisation of the embryonic gonad. (a) GFP^+ chicken PGC colonisation of the genital ridge in day 5 host embryos. (b) In the day 10 host GFP^+ donor chicken PGCs are distributed throughout the developing gonad. Bar 50mm.

3.3.7.2 Assessment of donor PGCs in the host adult gonad

GFP^+ cells from cell line 10-08-09 were injected into host wild type embryos (stage 16 HH). Embryos were cultured to hatch using the surrogate shell method by transfer post injection into phase III host shells (Perry 1988). The testes of sexually mature (more than 16 weeks post hatch) cockerels were examined (n=3 of 3), all testes were positive for GFP^+ cells (Figure 3.14b and c). The testes were sectioned and examined for the presence of GFP^+ cells. GFP^+ cells were localised in the

seminiferous tubules, of the gonad, situated next to the basement membrane (Figure 3.14c). In contrast,

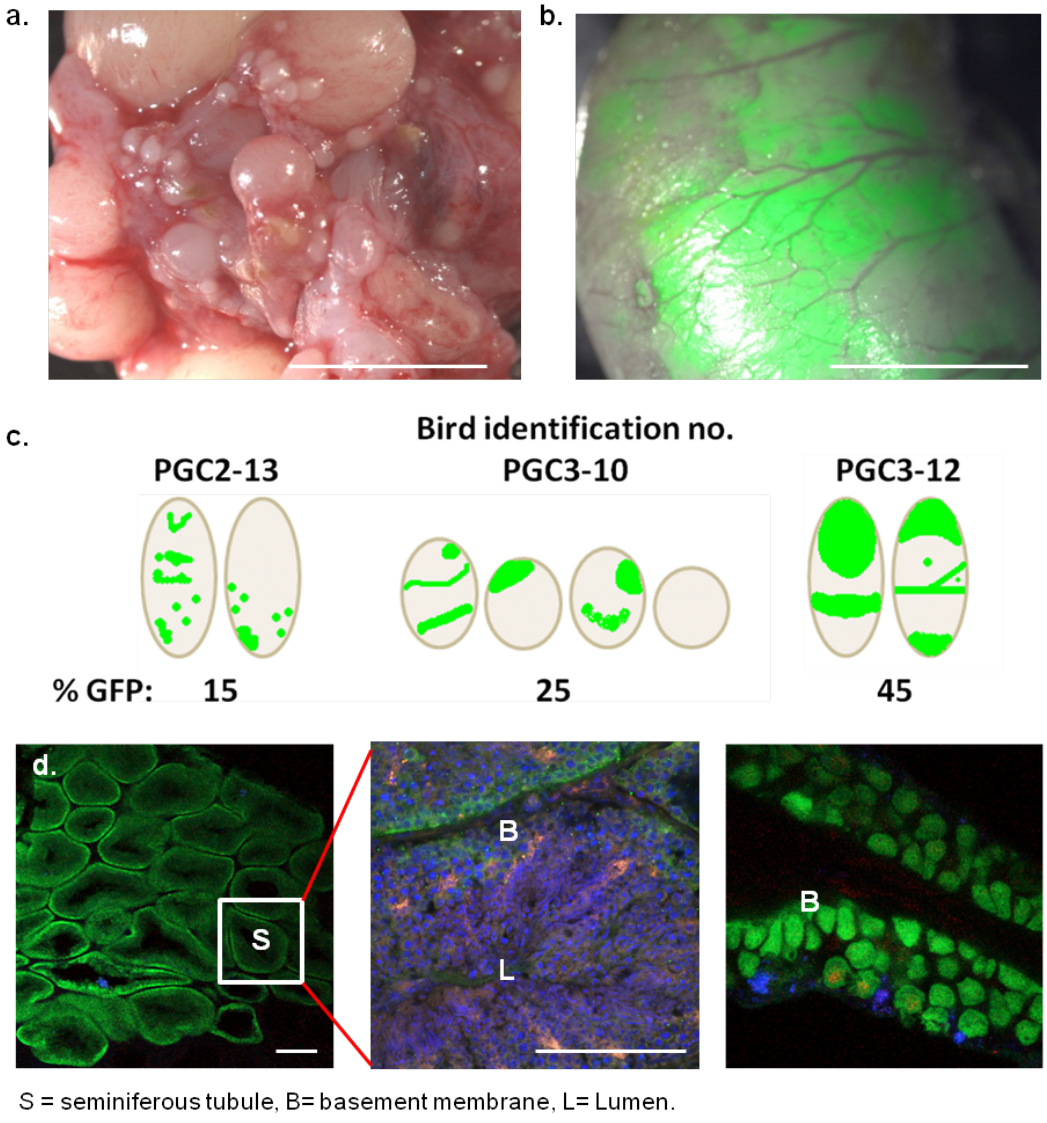


Figure 3.14 GFP+ PGC colonisation of the adult gonad. (a) Host hen ovary negative for GFP^+ tissue. (b) Host male testes with large areas of GFP^+ tissue. (c) Illustration of the amount of GFP^+ tissue in three sets of testes removed from male hosts. (d) Sections through the testes revealed large regions of GFP^+ cells. Bar 100mm. GFP^+ cells observed in sections throughout the mature gonad were located next to the basement membrane in the adult testes. Bar 200µm.

examination of the ovaries of female birds showed that although GFP^+ cells were present in the ovary cortex immediately after hatch, by the time the birds had reached sexual maturity GFP^+ cells could no longer be detected (n=3 of 3) (Figure 3.14a).

3.3.8 Assessment of cultured chicken PGCs ability to form functional gametes

To demonstrate that cultured male PGCs form functional gametes, wild type host embryos were injected with GFP^+ PGCs as before and raised to sexual maturity. The male GFP^+ PGCs (cell line 10-08-09), propagated in culture for 53 days were injected into stage 16 HH embryos (Materials and methods 2.5.4) and the embryos taken to hatch using the surrogate shell method (Perry 1988). A total of twenty-six embryos were injected twelve of which survived to hatch. A total of 10 hatchlings (7 males, 3 females) were successfully raised to sexual maturity (approximately 16 weeks for males, approximately 26 weeks for females). Semen from all seven male birds was collected by abdominal massage and genomic DNA extracted. The genomic DNA was then assessed using semi-quantitative PCR to estimate transgene contribution in the semen (Materials and methods 2.6.7).

FOUNDER BIRD ♂	EGGS SET	CHICKS HATCHED (%)	% GENOME EQUIVALENTS IN SEMEN	GFP⁺OFFSPRING (% transmission*)
PGC 2-13	242	147 (61%)	6	2 (2.8%)
PGC 3-6	242	83 (34%)	30	7 (16.8%)
PGC 3-11	190	110 (58%)	2	1 (1.8%)
PGC 2-3	-	-	1	-
PGC 3-5	-	-	4	-
PGC 3-10	-	-	1	-
PGC 3-12	-	-	1	-
FOUNDER BIRD ♀	**EMBRYOS EXAMINED**	**GFP⁺ EMBRYOS**		
PGC 2-2	63	0		
PGC 2-7	57	0		
PGC 3-3	68	0		

Birds highlighted in bold indicate embryos irradiated prior to injection.
* Transmission rate is adjusted for GFP allele heterozygosity and meiotic reduction.

Table 3.5 Frequency of germline transmission of donor GFP^+ chicken PGCs in host birds. Three potential germline chimeras were crossed with wild type stock birds and the offspring screened for GFP expression. GFP^+ chicks were fathered by all three birds, PGC2-13, PGC3-6 and PGC3-11. Transmission rates ranged from 1.8 to 16.8%. Irradiated birds showed the highest rates of transmission.

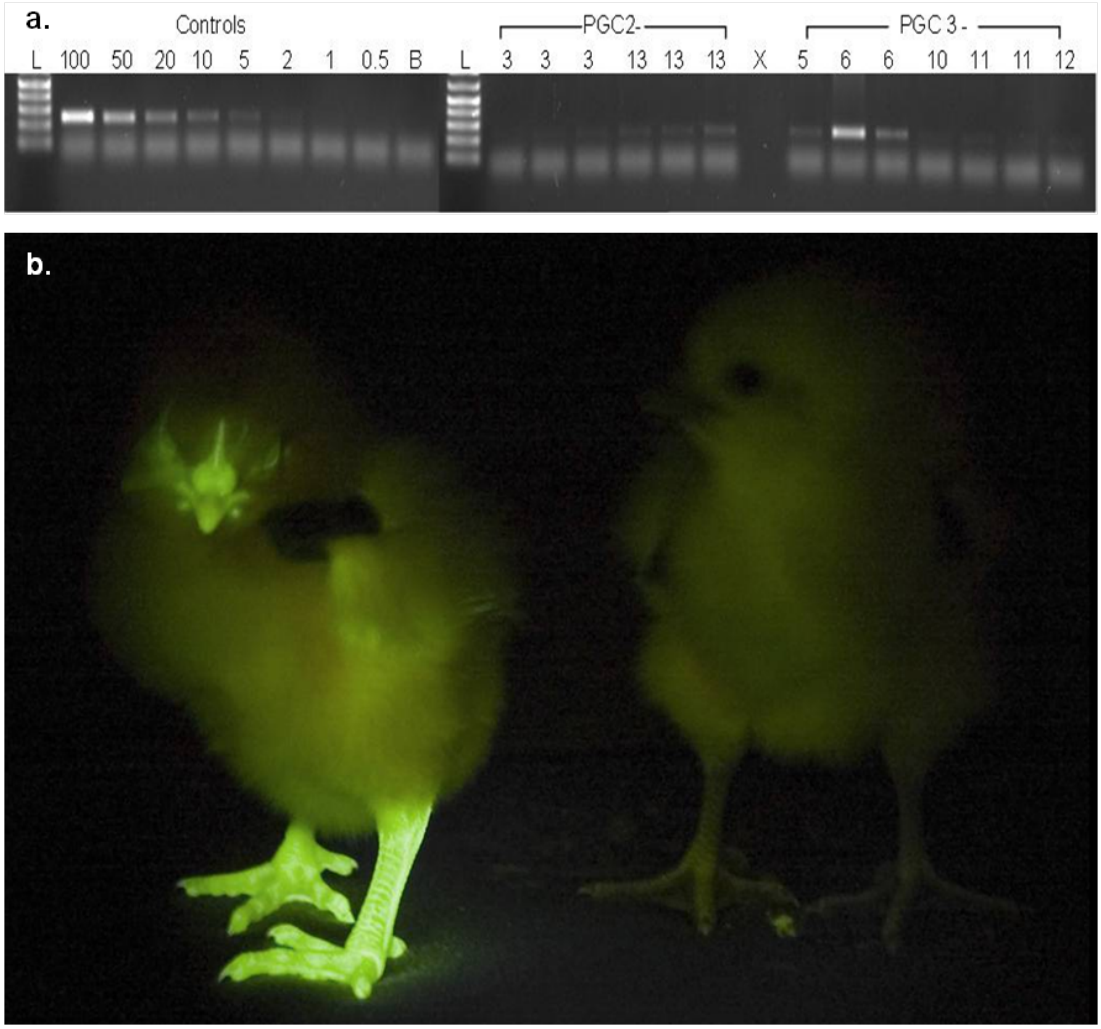


Figure 3.15 Chicken PGCs maintained in culture form functional sperm. Male embryos injected with cultured GFP$^+$ PGCs surviving to hatch were raised to sexual maturity. (a) PCR analysis of 13 samples of genomic DNA extracted from the semen of seven potential germline chimeras was screened. One, two or three semen samples were taken from each male and tested. Birds were identified as follows, PGC 2-3 (three samples). PGC2-13 (three semen samples), PGC 3-5 (one sample), PGC 3-6 (two samples), PGC 3-10 (one sample), PGC3-11 (two samples) and PGC3-12 (one sample). PCR was carried out on 50ng of genomic DNA extracted from semen. Copy number controls were set up in parallel using non-transgenic DNA spiked with varying amounts of vector DNA plasmid producing concentrations equivalent to one copy of the transgene, per genome (100%), per two genomes (50%); per five genomes (20%), per ten genomes (10%), per twenty genomes (5%), per fifty genomes (2%), per 100 genomes (1%) and per 500 genomes (0.5%). (b) Two G1 offspring from bird PGC2-13, the bird on the left is GFP$^+$ and therefore produced from a donor germ cell whist the bird on the right is not transgenic and therefore has been produced from PGC2-13's own germ cells.

Using GFP specific primers (Table 2.2) PCR was carried out on 50ng of genomic DNA in parallel with control PCR reactions. Copy number controls were set up using non-transgenic DNA spiked with varying amounts of vector DNA plasmid producing concentrations equivalent to one copy of the transgene, per genome (100%), per two genomes (50%); per five genomes (20%), per ten genomes (10%), per twenty genomes (5%), per fifty genomes (2%), per 100 genomes (1%) and per 500 genomes (0.5%) (Materials and methods 2.6.7). GFP sequence was detected in all the semen samples (Figure 3.15a) at frequencies ranging from 1 to 30% (Table 3.5). PGC 2-13 (6%), PGC 3-6 (30%) and PGC 3-11 (2%) were crossed with stock hens. All three male germ line chimeras fathered GFP^+ transgenic offspring (Figure 3.15b). As the GFP PGCs were heterozygous for the GFP allele and the semen is haploid, only half the actual transmission events were observed. Transmission efficiencies were adjusted to account for this and ranged from 1.8 to 16.8% (Table 3.5). Examination of testes from all chimeric cockerels (n=7 of 7), post mortem showed that GFP^+ cells were present in all birds. All three potentially germ line chimeric hens were crossed with stock cockerels and embryos screened. GFP fluorescence was not observed in any of the embryos resulting from these crosses (n=0 of 188).

3.4 DISCUSSION

3.4.1 Isolation and propagation of chicken PGCs

It has been demonstrated that it is possible to isolate chicken primordial germ cells that proliferate in culture for many months (van de Lavoir *et al*. 2006). Attempting to recapitulate the van de Lavoir method was difficult. As described in this chapter isolating chicken PGCs and establishing cell lines was not achieved when copying this method in its entirety. The initial step in establishing PGC cell lines was to aspirate blood from the vasculature stage 14-17HH embryos, the stage during which PGCs migrate from the germinal crescent via the vasculature to the genital ridge. It was demonstrated here that every blood sample aspirated from embryos at this stage of development does contain circulating PGCs (Figure 3.1). It can therefore be assumed that in the right culture conditions every culture initiated would have the potential to form a cell line. It was expected that by initiating cultures under the conditions of the van de Lavoir method that PGCs would be isolated with reasonable efficiency. As shown in these results this was not the case.

3.4.1.1 Manipulation of the van de Lavoir method can improve chicken PGC isolation.

As described the van de Lavoir medium for the culture of PGCs contains added growth factors, hFGF2 and SCF (species not stated). Using a simple experimental design it has been demonstrated that by changing the growth factors added to the medium it is possible to improve the efficiency of PGC isolation. By splitting aspirated blood samples containing circulating PGCs between medium with different added growth factor conditions; no added growth factors, hFGF2, mSCF and both hFGF and mSCF, it was possible to compare the efficiency of the different media (Table 3.3, Figure 3.3). The different culture conditions were marked as able to support isolation of the cells if three weeks post culture initiation there were 100 or more PGCs present in the culture well. The results presented in this chapter show that when only hFGF2 was added to the basic culture medium (Materials and methods 2.1.6) the frequency at which PGCs could be isolated from the blood was increased significantly compared to all other culture conditions tested including the

van de Lavoir method. The addition of mSCF to the cultures both in the absence of hFGF2 did not improve the culture conditions beyond that of hFGF2 alone. When both factors were added the presence of the mSCF appears to inhibit the effect of hFGF2 on isolation frequency. This suggests that mSCF may be having an inhibitory effect on chicken PGC survival. This conclusion is supported by the result observed showing that there was a decrease in isolation efficiency when mSCF alone was added to the culture medium compared to the no growth factor test group. This is supported by Choi *et al* (2010) who showed chicken PGC proliferation was not improved by the addition of hSCF to culture medium containing added hFGF2. SCF is a well-established factor for mouse PGCs (Matsui *et al*. 1991; Godin *et al*. 1991; Dolci *et al*. 1991; Pesce *et al*. 1993). The results presented here may reflect the effect of unknown levels of SCF in the culture medium as the BRL cells used to make conditioned medium and the STO feeder cells are both known to secrete growth factors LIF, SCF and IGF so additional SCF may not be required (Smith *et al*. 1988; Zsebo *et al*. 1990).

PGCs from chicken and mouse can be dedifferentiated in culture to form EG cells (Resnick *et al*. 1992; Matsui *et al*. 1992). Formation of mouse EG cellswas promoted by propagating mouse PGCs in medium with added FGF2 or stimulation of the AKT pathway. In chicken PGC cultures dedifferentiation to EG cells can occur spontaneously but can be further stimulated by removal of FGF2, SCF and sera from the culture medium. It was also observed here that in some of the established cultures, spontaneous formation of PGC-derived adherent cells was observed (Figure 3.5). None of the unsuccessful PGC isolations were assessed for the formation of EG cells during the three-week culture period. The reduction in isolation efficiency observed when culturing PGCs in medium with mSCF added might be attributed to increased induction of AKT signalling. As has been documented for mouse cultures an increase in AKT signalling may have resulted in a conversion of the chicken PGCs to EG cells that went undetected in this experimental analysis. It is also possible that an inhibitory effect may be associated with a lack of functional homology between species. If so this may have resulted in competitive inhibition of

the chicken c-kit receptor by the supplemented mSCF. Protein analysis would be required to verify this hypothesis.

3.4.1.2 SDF1 promotes chicken PGC isolation

The cytokine SDF1 is required for PGC migration, proliferation and survival in many vertebrates (Knaut *et al*. 2002; Molyneaux 2003; Takeuchi *et al*. 2009) and in PGC migration in the chicken embryo (Stebler *et al*. 2004). Although not a component of the van de Lavoir culture medium it was hypothesised that addition of SDF1 could improve isolation and propagation of chicken PGCs in culture. Analysis of the mRNA from the PGCs showed that the receptors for SDF1, CXCR4 and CXCR7 are expressed by chicken PGCs. This indicated that the PGCs would be able to bind to SDF1 if the cytokine were present in the culture medium. The number of PGCs in culture in the presence if mSDF1 increased significantly ($p<0.05$) more that PGCs cultured in the van de Lavoir culture mediumover the course of two weeks (Figure 3.8a). This suggests that as well as the documented role in migration of chicken PGCs that SDF1 may also promote cellular proliferation and survival.

SDF1 is not added to the van de Lavoir culture medium, however SDF1 may be present in a component of the medium. SDF1 activity in zebrafish is induced by interaction with CXCR4 not CXCR7. Given the different roles of the receptor ligand interaction the presence of SDF1 in the culture medium was assessed using the inhibitor AMD3100 that blocks the interaction of SDF1 and CXCR4 (Figure 3.8b). The results showed that when PGCs were cultured in the presence of AMD3100 the number of cells in the culture well increased at the same rate as the control cells (van de Lavoir method) for the first seven days. However during the next seven days the cell number in the inhibitor treated wells did not increase. This showed that that SDF1 was present in the culture medium and it was being inhibited by the presence of AMD3100. This indicated that SDF1 does have a function in the survival and proliferation of chicken PGCs *in vitro*.

When the addition of SDF1 to culture medium was assessed for its ability to improve isolation of the chicken PGCs from embryonic blood, the frequency of isolation was improved (Figure 3.9a). The effect was most pronounced when SDF1 and hFGF2 were both added to the culture medium. It was also observed that if the amount of SDF1 added was reduced by a half (25ng/ml to 12.5ng/ml) the isolation frequency improved. This indicated that addition of SDF1 was more effective at concentrations lower than initially used. Despite improvements in isolation frequency it was also observed that the addition of SDF1 to the culture medium resulted in a change in the chicken PGC morphology (Figure 3.9c). This may be due to the interaction of SDF1 and its second receptor CXCR7. The function of CXCR7 is to sequester SDF1 when present at high levels SDF1 *in vivo* (Mahabaleshwar *et al*. 2008; Naumann *et al*. 2010). It is perhaps possible that due to role SDF1 plays in migration *in vivo* that surface bound SDF1 resulted in an attraction between the cells. If CXCR7 binds a different site on the SDF1 than CXCR4 this may result in competition between receptors on two cells for the same SDF1 molecule. This could result in a bringing together of cells to form floating colonies.

3.4.2 Expression of pluripotency genes in chicken PGCs

Gene analysis was used to identify genes differentially expressed between ES cells and PGCs in mouse to gain a better understanding of why PGCs are germline restricted. Analysis of PGC gene expression in mouse embryos showed that mouse PGCs express pluripotency markers; *Oct4*, *Nanog* and *Sox2* whilst mouse ES-cells express these three genes and *c-Myc* and *Klf4*. When mouse PGCs dedifferentiate to form EG cells, it coincides with initiation of expression of *c-Myc* and *Klf4* (Durcova-Hills *et al*. 2008). This suggested that a lack of *c-Myc* and *Klf4* expression restricts PGCs to the germ cell lineage. Analysis of the chicken ES cells showed that like mouse ES cells, they too express all five marker of pluripotency. When analysis of the mRNA from the established chicken PGC lines was carried out it was hypothesised that they would express the same genes as the mouse PGC. However, the results showed that as well as expressing *cPouV* (*Oct4*), *cNanog* and *cSox2* (Figure 3.11) the PGCs also expressed *c-Myc* and *Klf4* (Figure 3.12). This result

suggests that in the case of chicken PGCs restriction to the germline is not due to the absence of pluripotent genes but instead may be determined by yet to be identified maternally derived proteins and a germline determined by preformation. This potential difference in germline formation, epigenesis in mouse and most likely preformation in chicken may account for the difference in PGC gene expression identified between the two species. This hypothesis could be confirmed if examination of PGC gene expression in other vertebrates whose germ line is preformed, such as zebrafish, medaka fish and Xenopus, showed the same gene profile to the one documented here for chicken PGCs. Although the cultured cells were validated to be PGCs based on morphology (Figure 3.2a) and *CVH* expression (Figure 3.6c) an *in vitro* culture effect cannot be dismissed. Analysis of the nine-day gonad as a representation of chicken PGC gene expression *in vivo* would not be suitable given that *c-Myc* and *Klf4* are expressed in many somatic tissues during chick embryonic development.

3.4.3 Germline transmission of donor male PGCs in male hosts

Van de Lavoir *et al* (2006) and Choi *et al* (2010) have demonstrated that chicken PGCs maintained in culture beyond forty days were still able to colonise the gonad and form functional sperm. Here it was demonstrated that PGCs derived from a third breed of layer chicken could be isolated from embryonic blood, proliferate in culture for more than fifty days whilst retaining the ability to form functional gametes in a host gonad. All the PGCs isolated and propagated long term here were identified to be male. These findings fit with what was documented by van de Lavoir *et al* (2006) where it was reported that only two of the fourteen cultures established were female and that unlike the male counterparts these chicken PGCs could not be maintained in culture beyond 77 and 109 days. PGC transplants to opposite-sex recipients in mouse have shown that whilst male PGCs in a female host will form functional oocytes female PGCs in a male host do not (Ford *et al*. 1975; Kocer *et al*. 2009). In chickens, cultured PGCs have not been demonstrated to form functional gametes in the opposite-sex hosts (van de Lavoir *et al*. 2006). This data supports what was observed here when the male PGCs isolated from a single GFP expressing embryo were

shown to migrate to and colonise the developing gonad when injected into hosts (Figure 3.13b). The chicken PGCs were observed in the female embryonic gonad at day 10 (Figure 3.13c) but not in the ovary of adult (Figure 3.14a). GFP expressing cells were observed in the testes of sexually mature male recipients (Figure 3.14). All the male birds produced were shown to be chimeras by the presence of the GFP transgene in genomic DNA extracted from their semen (Figure 3.15a). This was further validated when the chimeric male birds fathered chicks with the donor PGC phenotype (GFP^+, Figure 3.15, Table 3.5), confirming that donor chicken PGCs had formed functional sperm. Conversely none of the three female putative chimeras transmitted the donor PGC phenotype. The male PGCs were therefore unable to form functional gametes in the female ovary as would be expected from the work mentioned previously.

3.4.4 Comparison of achieved transmission rates

When the rates of transmission achieved here were compared to those recorded by van de Lavoir *et al* (2006) and Choi *et al* (2010) they were lower, 1.8% to 16.8% compared to over 80%. In the case of van de Lavoir *et al* (2006) the difference in transmission rates could have been attributed to a higher number of chimeras tested, forty-three compared to only five tested in this study. However, Choi *et al* (2010) only tested five chimeras using cells that had been in culture for a similar period of time to the cells used in the experiments documented here, just over fifty days. These findings suggest that there may be a difference in the culture conditions that alter the PGCs ability to form functional sperm. As GFP expressing PGCs were used in these experiments and wild type PGC were used by Choi *et al* (2010) it could be suggested that the expression of GFP may have an adverse effect. However the chicken line from which these PGCs have been established do not display any differences in fertility compared to their wildtype counterparts. Alternatively the differences in transmission efficiency may be sex-related, as four out of the five G0 birds tested by Choi *et al* (2010) were female.

3.5 CONCLUSIONS

A number of conclusions can be drawn from the experiments outlined in this chapter. Firstly the method outline by van de Lavoir *et al* (2006) was not easily recapitulated but did provide the basis for establishing chicken PGCs cultures *in vitro*. It was shown here that unlike what was set out in the published method SCF may not be a required addition to the PGC culture medium. It was also demonstrated here that the addition of SCF from mouse might be having an inhibitory effect on the isolation of the PGCs from embryonic blood. Without definitively knowing from what species the SCF used in the van de Lavoir method was derived it is difficult to make comment. However it is thought that the SCF added in the published protocol is from chicken and not commercially available.

It was shown that chicken PGCs express both receptors of SDF1 and although addition of the mitogen to cultured medium increased PGC proliferation it also resulted in a change in morphology. From the observed results it was concluded that if SDF1 is used for the initial derivation of the PGCs it should then be removed from the culture medium for continued propagation of the cells.

Although the chicken PGCs derived were shown to form functional gametes, the rates of transmission were lower than observed by either van de Lavoir *et al* (2006) or Choi *et al* (2010). However, it cannot be concluded that this was as a result of differences in the culture conditions and so further analysis of the culture methods used here and in the published work would be required.

CHAPTER 4: INVESTIGATION OF SIGNALLING PATHWAYS IMPLICATED IN PGC PROLIFERATION AND SURVIVAL *IN VITRO*

4.1 INTRODUCTION

A feeder and serum free system for the culture of mouse ES cells has been established (Ying *et al*. 2008). From the first cultures of mouse ES cells until quite recently maintenance of these cells in culture has required a complex combination of conditioned medium, serum extracts, hormones, cytokines and growth factors (Evans and Kaufman 1981; Yoshida *et al*. 1994; Ying *et al*. 2003; Sato *et al*. 2004). The establishment of a feeder and serum free culture system for ES cells allows for research to be standardised and much more reproducible. As has been observed in ES cell culture a major source of variation in the success and reproducibility for establishing chicken PGC lines is the requirement of serum and feeder cells in the culture system. In the van de Lavoir culture method a mouse cell feeder layer, added chicken serum, FBS (foetal bovine sera) and medium conditioned on BRL (buffalo rat liver) cells are all required, each of these components provides a large source of variation. In addition to this recombinant growth factors are added to the medium or secreted into the culture medium from the feeder cells and the BRL cells. The BRL cells secrete LIF into the conditioned medium (Smith *et al*. 1988) and STO mitotically inactivated feeder cells secrete mouse LIF, SCF, and IL6. The exogenous factors provided by the chicken sera and the FBS are undefined but likely to contain undefined levels of FGF2, SCF, IGF1 and other growth factors.

4.1.1 The role of extracellular signals in PGC survival and proliferation

Several key growth factors, FGF2, SCF, LIF, IGF1, have been shown to key in the survival and proliferation of PGCs both *in vivo* and *in vitro*. Investigation of germ cell development in several species has shown that activation of several pathways, PI3K/AKT, MEK/ERK and JAK/STAT, mediated by the presence of these growth factors, are essential for germ cell migration, proliferation and survival. The

PI3K/AKT, MEK/ERK and JAK/STAT pathway are also implicated in the *in vitro* culture of PGCs. The PI3K/AKT pathway inhibits apoptosis and MEK/ERK signalling promotes cellular proliferation. The JAK/STAT pathway has been linked to dedifferentiation of mouse PGCs grown in culture.

4.1.1.1 The PI3K/AKT pathway

Activation of the PI3K/AKT pathway, initiates a signalling cascade that promotes AKT expression and in turn inhibition of apoptosis (Figure 1.6). Over-expression of AKT in mice results in increased PGC proliferation *in vivo* (De Miguel *et al*. 2002) but when the expression is inhibited using PI3K inhibitors no effect was observed on the mouse PGC cultures *in vitro* (De Miguel *et al*. 2002). The PI3K/AKT pathway can be activated by a number of factors including; IGF1 (Kulik *et al*. 1997; Reindl *et al*. 2011) and via SCF/cKit interaction (Sette *et al*. 2000; De Felici 2000). SCF has been demonstrated to be required for mouse PGC cell survival *in vivo* and *in vitro* (Dolci *et al*. 1991; Pesce *et al*. 1993; Sette *et al*. 2000). IGF1 an activator of the PI3K/AKT pathway promotes germ cell survival, proliferation and migration in the zebrafish embryo (Schlueter, Sang, *et al*. 2007). When IGF1 expression is inhibited in zebrafish embryos the PGCs fail to migrate appropriately, resulting in fewer PGCs colonising the gonad (Schlueter *et al*. 2007; Sang *et al*. 2008). Van de Lavoir *et al* (2006) added SCF to culture medium to derive and maintain chicken PGCs and showed that when both SCF and FGF2 supplementation was ceased adherent PGC grew out as EG cell populations. The affects, of SCF and other components of the medium, on PI3K/AKT signalling, in chicken PGCs, have not been previously investigated.

4.1.1.2 The MEK/ERK pathway

The MEK/ERK signalling pathway is involved in cellular proliferation and survival. After activation by external cues that promote a MEK1/2 signalling cascade, MEK/ERK activation culminates in inducing cytoplasmic and transcription factors (Figure 1.7). Defects in MEK/ERK signalling can result in uncontrolled cell growth associated with many cancers (Wong 2009). This pathway has been demonstrated to

both promote and inhibit differentiation of mouse ES cells (Burdon *et al*. 1999) and human ES cells (Li *et al*. 2007). When the MEK/ERK pathway is inhibited mouse PGCs fail to migrate and show a reduction in proliferation *in vitro* (De Miguel, 2002; Farini *et al*. 2007). MEK/ERK signalling can be activated by FGF2 binding to FGFRs (Campbell *et al*. 1995). Addition of FGF2 to PGC cultures increases proliferation of both mouse and chicken cells (Resnick *et al*. 1992; Choi *et al*. 2010) but when FGF2 supplementation is maintained for longer than five days in mouse cultures, it results in embryonic germ (EG) cell formation (Resnick *et al*. 1992). Van de Lavoir *et al* (2006) recommended the addition of hFGF2 to chicken PGC culture medium but Choi *et al* (2010) showed that removal of hFGF2 for a period of 24hrs had little effect on gene expression and did not affect normal migratory behaviour of the chicken PGCs *in vitro*. These data and the demonstration in chapter 3 that chicken PGCs could be derived in the absence of additional FGF2 suggests that more than one source of FGF2 or other activators of MEK/ERK are present in the culture medium.

4.1.1.3 LIF and the JAK/STAT pathway

The JAK/STAT pathway has not been extensively studied in the germ cells. It is known that activated STAT3 maintains mouse ES cells in an undifferentiated state (Niwa *et al*. 1998) whilst it promotes differentiation of mouse spermatagonial stem cells (SSC) (Oatley *et al*. 2010). In mouse PGCs, activation of STAT3 promotes dedifferentiation to EG cells (Durcova-Hills, 2008). Activation of this pathway can be induced by LIF binding to the gp130 receptor homeodimer (Koshimizu *et al*. 1996) (Figure 1.9). LIF was identified as the polypeptide present in BRL-conditioned medium conditioned that inhibits differentiation (Robertson 1986; Smith *et al*. 1988). Addition of purified LIF to cell culture medium promotes survival and self-renewal in mouse ES cell cultures (Farini *et al*. 2005) most likely through activation of JAK/STAT. In PGC culture the addition of LIF can result in EG cell formation in a number of species (Matsui *et al*. 1992; Park and Han 2000; Kakegawa *et al*. 2008). In contrast, LIF supplementation *in vitro* has been shown to inhibit mouse PGCs from entering meiosis (Chuma and Nakatsuji 2001; Farini *et al*. 2005). There is also

conflicting evidence for the role of LIF on PGCs *in vivo*. It has been observed that when LIF induced signalling is compromised it results in a reduction of PGCs colonising the gonad however this has no overall effect on fertility (Ware *et al*. 1995; Molyneaux 2003). These results suggests that LIF might only be important in the *in vitro* propagation of germ cells and that the levels of LIF in the culture system may have to be maintained within tight limits to avoid formation of EG cells. LIF can also stimulate activation of the ERK/2 pathway (Yoshida *et al*. 1994) and FGF2 has also been shown to stimulate increase LIF expression in feeder cells (Rathjen, Toth, *et al*. 1990; Rathjen, Nichols, *et al*. 1990)

4.1.1.3 Complexities of the van de Lavoir culture medium

As mentioned previously, the components of the van de Lavoir culture medium required for the propagation of chicken PGCs *in vitro* are complex and varied. For these reasons a comprehensive understanding, of the growth factors involved in the survival and proliferation of PGCs will be invaluable to establishing a serum and feeder free culture medium. This would be useful for the recapitulation of chicken PGC culture in different laboratories and improve chicken PGC based research. To address this issue the status of the three signalling pathways discussed above were investigated, in chicken PGCs *in vitro*. To examine the role each pathway plays in the survival and maintenance of chicken PGC cultures small molecule inhibitors were added to cells *in vitro* and the effect on cell proliferation assessed.

Many growth factors and cytokines from several vertebrate species are likely to be present in the van de Lavoir culture medium. This includes LIF, IGF1 and SCF from rat, and mouse excreted from BRL and STO cells, purified human FGF2 and mouse SCF, FGF2 and IGF etc from the chicken and bovine sera. To reduce the complexity and gain understanding of what chicken PGCs require for propagation in culture analysis of signalling pathways and the factors that activate them is essential. Signalling pathways can be activated by direct and indirect mechanisms and signalling between pathways increases the complexity. Given the complexity of signalling pathways and the undefined levels of growth factors and cytokines present

in chicken PGC culture medium a series of induction experiments investigating the individual elements of the culture medium were carried out. The aim of these experiments was to identify which of the three signalling pathways were activated by what components of the medium. By identifying these components it is hoped that a better understanding of the factors required for the maintenance of chicken PGCs *in vitro* will be achieved.

4.2 AIMS

1 To identify the roles of the PI3K/AKT, MEK/ERK and JAK/STAT pathways in germ cell proliferation and survival *in vitro*.

2 To identify components of the culture medium that induce activation the PI3K/AKT pathway.

3 To identify components of the culture medium that induce activation the MEK/ERK pathway.

4 To identify components of the culture medium that induce activation the JAK/STAT pathway.

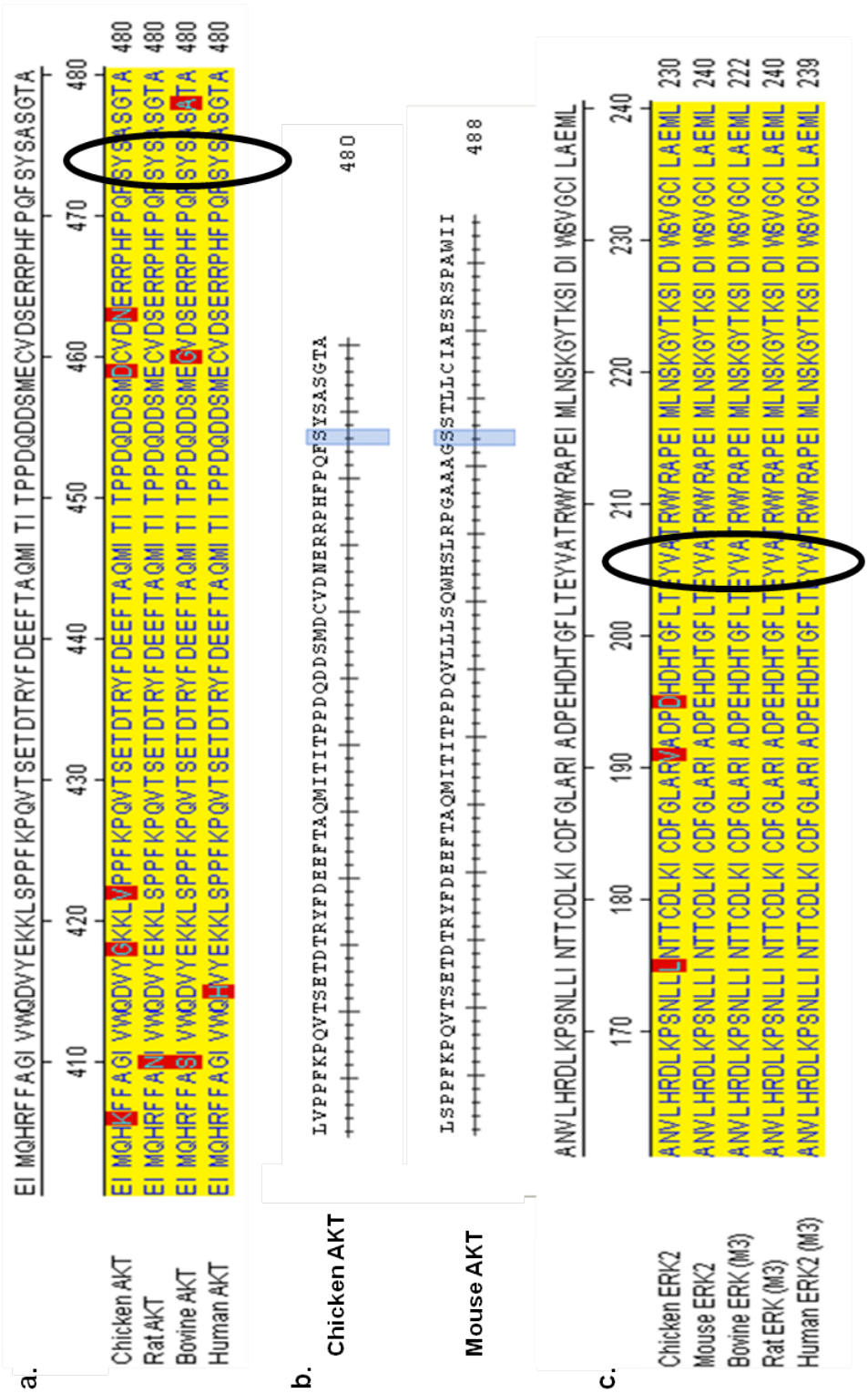
4.3 RESULTS

4.3.1 Determination of signalling pathways involved in chicken PGC proliferation *in vitro*

4.3.1.1 Choice and validation of antibodies

Before any experiments were carried out to investigate the role of pathways, PI3K, ERK1/2 and JAK/STAT antibodies that would identify pathway activity had to be chosen and validated. Three antibodies, phospho-AKT (Ser473), (phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) and phos-STAT3 (Tyr705), which had been used to identify activation of the three pathways in mouse were recommended by H Murray. Each antibody functions by detecting endogenous levels of the particular protein when phosphorylated at specific residues. Neither the AKT nor the ERK1/2 antibodies were reported by the manufacturer to have cross-reactivity with chicken. To determine if all three antibodies could be used to identify phosphorylation of the endogenous proteins the polypeptide sequences from chicken, mouse, rat, human and bovine for AKT, ERK1/2 and STAT3 were assessed (Figure 4.1). Alignment of these sequences showed that in all sequences the antibody target residue was conserved between the five species. To validate this experimentally total cellular protein was extracted from three chicken PGC lines, 03.08.09, 08.08.09 and 10.08.09 (Materials and methods 2.6.9) that had been cultured in basic medium plus hFGF2. Protein from an induced mouse ES (mES) cell was provided as a positive control (H Murray). Western blot analysis was then carried out to identify if the chosen antibodies could detect the phosphorylated proteins (Figure 4.2). Each PGC sample was tested in triplicate at three different volumes of cell lysate, 4, 8 and 12µl. Both the AKT and the ERK1/2 antibodies clearly detected the phosphorylated forms of their target proteins in all the chicken samples and the positive mES cell control. This was clear validation that both the AKT and ERK1/2 antibodies were suitable for detection of protein in chicken samples. When the STAT3 antibody was tested only very low levels were detected in both the chicken and mouse samples. Although this did no validate the STAT3 protein as effective for use with chicken samples it is demonstrated later that STAT3 can be detected using this antibody. With the lack of activated STAT3 detected in the mouse sample it was considered that the antibody

may have required replacing and a new aliquot was ordered prior to the experiments that are presented in 4.3.2.3.



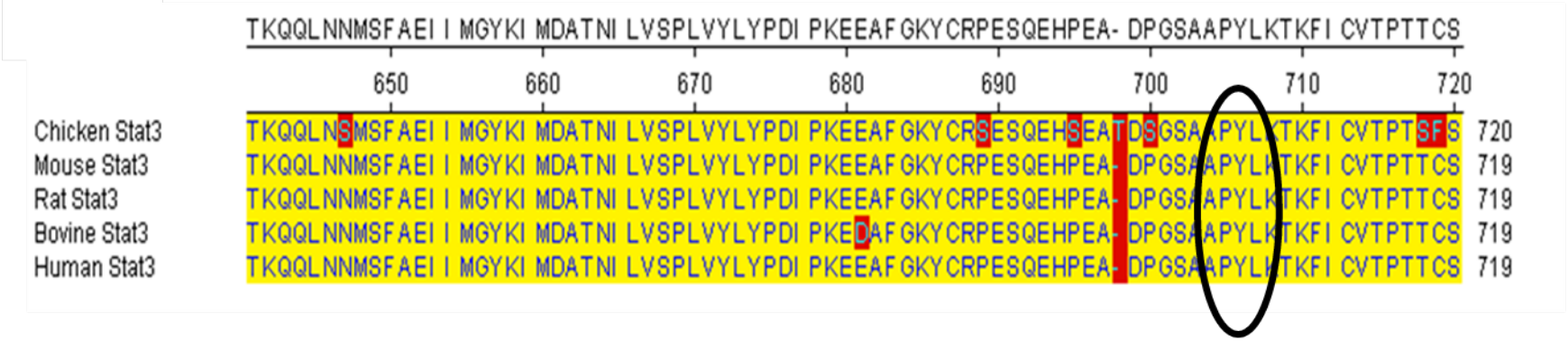


Figure 4.1 Comparison of vertebrate polypeptide sequences for AKT, ERK and STAT3. AKT is 90% conserved between (a) chicken, rat, bovine and human and (b) between chicken and mouse. The antibody phosAKT (SER472) (Cell Signalling) detects endogenous levels of AKT only when phosphorylated at serine 472 (ringed in black, a.; highlighted in blue, b). This residue is conserved between the species. (c) The alignment shows that ERK1/2 is over 90% residues are conserved between chicken, mouse, bovine, rat and human. The antibody phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cell Signalling) detects endogenous levels of ERK1/2 when phosphorylated at the threonine 202 and tyrosine 204, these residues are conserved between species as highlighted (black ring). (d) STAT3 sequences from chicken, mouse, rat, bovine and human have been aligned and show over 97% conservation of residues between the species, the alignment shows a section of the STAT3 sequence. The antibody phospho-STAT3 (Tyr705; Cell Signalling) detects endogenous levels of STAT3 when phosphorylated at tyrosine 705. This residue is conserved as highlighted (black ring). In the alignment the methionine residue at position one, which is cleaved during protein synthesis is present so the phosphorylated residues are situated one position further to the right. Conserved residues are highlighted in yellow.

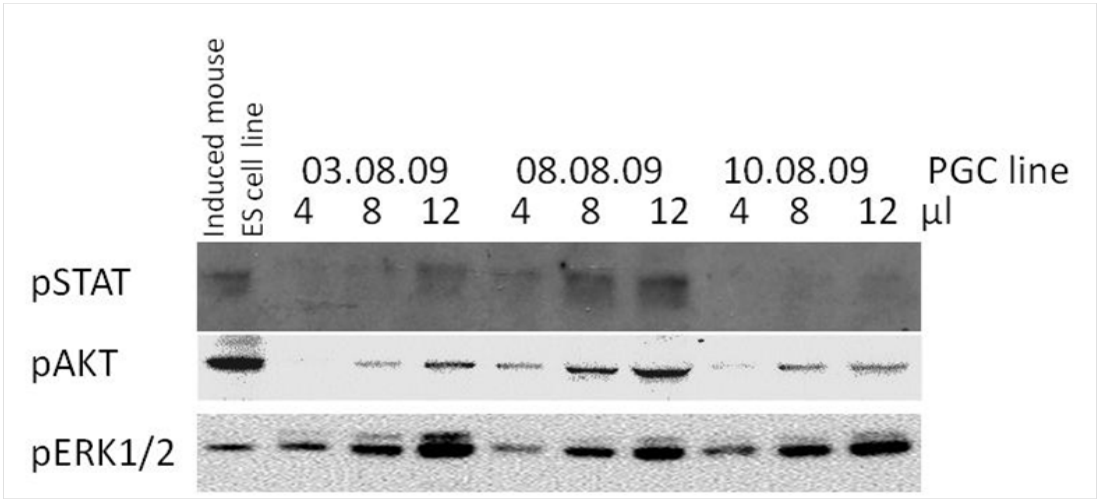


Figure 4.2 Western blot analysis of three PGC cell lines. Western analysis of total protein from chicken PGCs from three lines, 03.08.09, 08.08.09 and 10.08.09 grown in basic medium plus hFGF2 and an induced mouse EScell line. Three different volumes of protein were used and the membrane bound protein was probed for phosphorylated STAT3, phosphorylated AKT and phosphorylated ERK1/2 to show that the antibodies used could detect chicken proteins.

4.3.1.2 The effect of PI3K inhibition on PGC proliferation *in vitro*

The PI3K/AKT signalling pathway is associated with cell growth and survival. Activation of PI3K/AKT signalling has been shown to inhibit apoptosis in cell that were exposed to high levels of UV (Kulik *et al*. 1997). LY294002 is a PI3K specific inhibitor (Vlahos *et al*. 1994, Feng *et al*. 1998) that has been used effectively to inhibit PI3K/AKT pathway activation in a neurons resulting in increased apoptosis (Yao and Cooper. 1995). The effects of LY294002 mediated inhibition on PI3K mediated signalling on chicken PGCs *in vitro* was assayed (Figure 4.3a). Chicken PGCs from three cell lines, 08-08-09, 03-10-10 and 06-10-10, were grown in basic culture medium plus hFGF2 (2ng/ml) (Materials and methods 2.7.3). DMSO vehicle (control) or inhibitor LY294002 (10µM) dissolved in DMSO was added to the cultures and after seven days the effects on cell number were assessed (Figure 4.3b). Each cell line was assayed three to six times in three separate experiments. Experiments were carried out in both the presence and absence of STO feeder cells. After seven days viable cells, indentified from dead cells using trypan blue dye (Materials and methods 2.7.4), were counted. Approximately 90% of cells in the

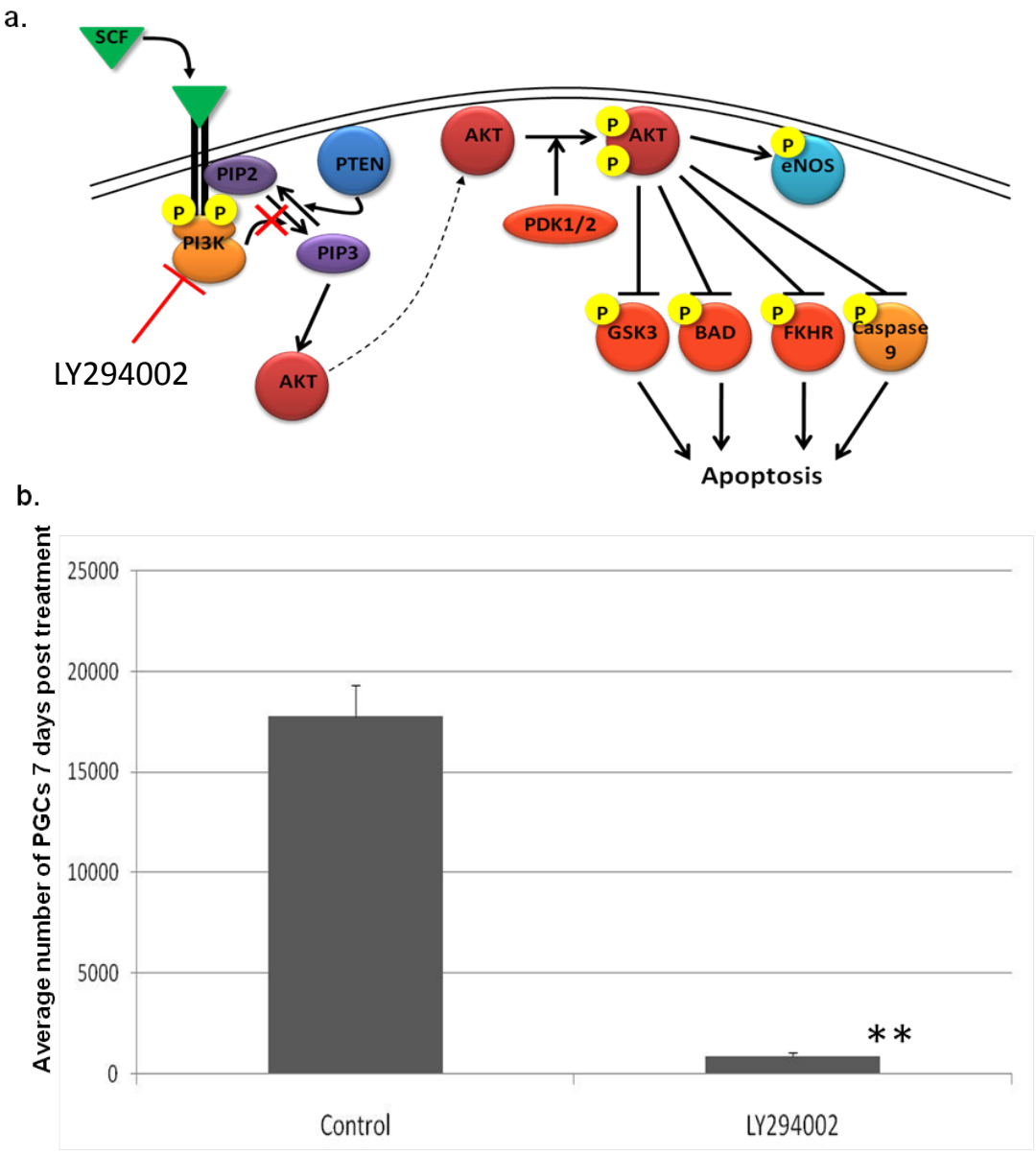


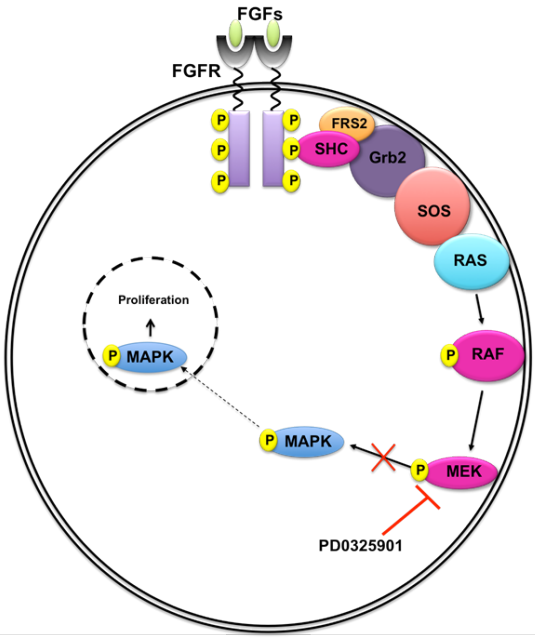
Figure 4.3 The effect of LY294002 on PGC proliferation *in vitro*. (a) PI3K inhibitor LY294002 blocks SCF-induced activation of AKT. When SCF binds the KIT receptor and signals for PI3K to bind to the receptor LY294002 blocks phosphorylation of PI3K. Unphosphorylated PI3K cannot induce conversion of PIP2 to its second messenger PIP3, resulting in AKT not being activated. Unphosphorylated AKT can no longer inhibit inducers of apoptosis, resulting in increased cell death. (b) Chicken PGCs from three lines, 08.08.09, 03.08.09 and 06.10.10 were were treated with either DMSO (Control) or LY294002. Each cell line was assayed 3-6 times in three separate experiments. Wells were seeded with 1000 chicken PGCs 24 hrs prior to addition of the individual treatments. After 7 days cell viability was assessed by trypan blue staining. The average number of cells was calculated for each treatment group. Error bars, S.E.M. **, $P<0.01$.

LY294002 treated wells were trypan blue positive (dead/dying) in comparison to fewer than 10% of cells in DMSO treated wells. The comparison of total cell number after seven days showed that significantly more ($P<0.01$) cells were present in the DMSO treated wells than in the LY294002 treated samples. This difference in total cell number between control and treated samples indicated a severe inhibition of PGC proliferation resulting from the addition of LY294002 to the culture medium. No significant difference was observed in the samples grown in the presence or absence of feeder cell layer. This indicated that the reduction in chicken PGC proliferation was caused by a direct effect of inhibitor on the PGCs and a not secondary effect resulting from changes in the feeder layer. As PI3K is inhibited by LY294002 these results suggest that PI3K mediated signalling is essential for PGC propagation *in vitro*.

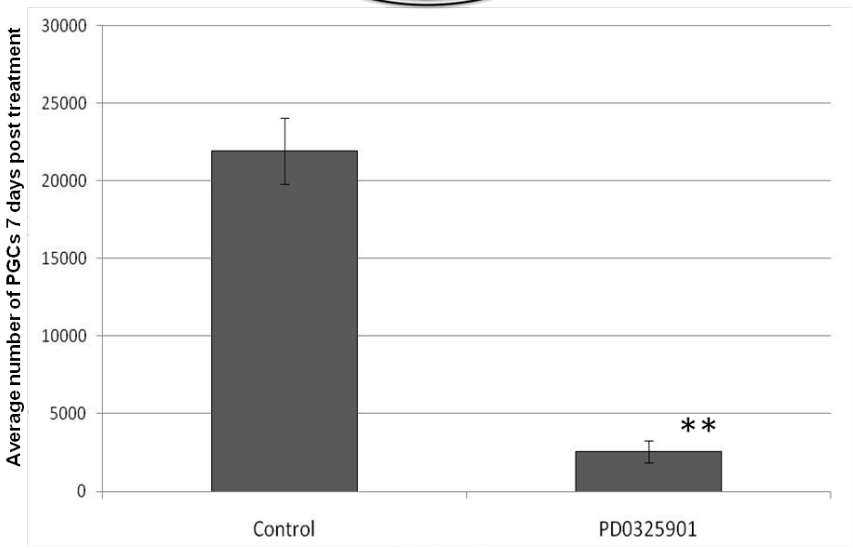
4.3.1.3 The effect of MEK inhibition on PGC proliferation *in vitro*

Extracellular growth factors, such as FGF2 activated intracellular targets through a cascade of signalling mediated by the MEK/ERK pathway. MEK/ERK signalling plays an important role in the regulation of cellular proliferation and apoptosis. PD0325901 is a potent inhibitor of MEK1 and 2 (Thompson and Lyons, 2005) and has been demonstrated to efficiently reduce proliferation in carcinoma cells (Henderson *et al*. 2010, Haura *et al*. 2010). PD0325901 mediated inhibition of MEK was assessed for its effect on PGC propagation *in vitro*. As in subsection 4.3.1.2 PGCs from three separate cell lines; 08-08-09, 03-10-10 and 06-10-10; were grown in basic culture medium plus hFGF2 to which DMSO vehicle (control) or PD0325901 (3ug/ml) dissolved in DMSO was added (Materials and methods 2.7.3). Each cell line was assayed three to six times in three separate experiments both in the presence or absence of STO feeder cells. After seven days the total number of live chicken PGCs per well was counted and compared between treatment and control samples. Cell viability was assessed using Trypan blue dye staining (Materials and methods 2.7.4). Approximately 90% of cells in the PD0325901 treated wells were trypan blue positive (dead/dying) compared to fewer than 10% of cells in DMSO

a.



b.



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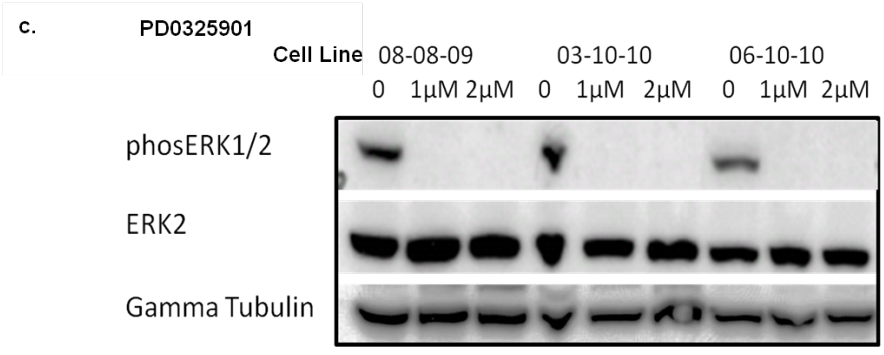

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Figure 4.4 The effects of PD0325901 on proliferation of PGCs *in vitro*. (a) When FGF-2 binds to its receptor a signalling cascade is initiated, leading to phosphorylated MEK inducing phosphorylation of ERK1/ERK2 (MAPK). PD0325901, a non-competitive ATP inhibitor, blocks MEK activated phosphorylation of ERk1/2 (MAPK) thus blocking pathway signalling. (b) Chicken PGCs from three lines, 08.08.09, 03.08.09 and 06.10.10 were treated with either DMSO (Control) or PD0325901. Each cell line was assayed 3-6 times in three separate experiments. Wells were seeded with 1000 chicken PGCs 24 hrs prior to addition of the individual treatments. After 7 days trypan blue staining was used to assess cell viability. The average number of cells was calculated for each treatment group. Error bars, S.E.M. **, $P<0.01$ (b) Western analysis of total protein from chicken PGCs from three lines, 08.08.09, 03.08.09 and 06.10.10 treated with 1μM and 2μM of PD0325901 for 5 hours. Untreated chicken PGCs from each of the three lines were used as a control, marked as 0 on blot. The membrane bound protein was probed for phosphorylated ERK1/ERK2, total ERK2 and gamma tubulin. Cells treated with the inhibitor showed a complete depletion of phosphorylated ERK1/2. All samples were positive for ERK2 protein. Gamma tubulin shows that the protein was similar between samples.

treated wells. Total cell number after seven days was averaged and presented in figure 4.3b the results showed that there were significantly ($P<0.01$) more cells in the DMSO treated wells than in PD0325901 treated cultures. These results indicate that the presence of PD0325901 in culture medium is inhibiting proliferation of chicken PGCs *in vitro*. No significant difference was observed in the samples grown in the presence or absence of feeder cell layer indicating that the reduction in chicken PGC proliferation is a direct effect of PD0325901 and not due to secondary effects resulting from changes in the feeder layer resulting from the inhibitor treatment.

4.3.1.4 Assessment of ERK1/2 phosphorylation in PGCs when cultured in the presence of MEK inhibitor PD0325901

To confirm that the reduction in chicken PGC proliferation resulted from PD0325901 inhibition of MEK the phosphorylated status of ERK1/2 was assessed by western blot analysis. Three chicken PGC lines, 08-08-09, 03-10-10 and 06-10-10 were grown in the presence of the inhibitor at 3μg/ml and 6μg/ml for five hours. These concentrations were used to recapitulate the concentration used in the proliferation experiments (3μg/ml) and to account for the reduction in the time the cells were exposed to the inhibitor (6μg/ml). After incubation total protein was extracted from the PGCs (Materials and methods 2.6.9). The proteins resolved on a 12% acrylamide gel and transferred to Hybond PVDF membrane. The membrane-

bound proteins were then probed using the antibody p44/42 (Erk1/Erk2) (Table 2.1) that detects ERK1/2 when phosphorylated at residues, threonine 202 and tyrosine 204. As shown in figure 4.4c phosphorylated ERK1/2 was detected in the control samples, PGCs cultured in basic medium plus hFGF2. However after culture in the presence of PD03255901 the pathway is completely down regulated, evidenced by a complete absence in ERK1/2 phosphorylation (figure 4.4c). The membrane was then reprobed (Materials and methods 2.6.9) using an antibody that detects ERK2 total protein. This result showed that ERK2 protein, with the potential to be phosphorylated was present in all samples. An antibody specific to chicken gamma tubulin was used to shows that the amount of protein used was similar between samples. This result confirms that addition of PD0325901 to culture medium results in a complete down regulation of the MEK/ERK pathway.

4.3.1.5 The effect of JAK inhibition on PGC proliferation *in vitro*

Activation of the JAK/STAT pathway has been implicated in the dedifferentiation of mouse PGCs into EG cells (Durcova-Hills *et al*. 2008). To investigate the role this pathway plays in chicken PGC proliferation a JAK inhibitor, 420099 was used to block activation of the pathway (Figure 4.5a). PGCs from two lines; 06.10.10 and 08.08.09 were grown in basic culture medium plus hFGF2 containing DMSO vehicle (control) or inhibitor 420099 dissolved in DMSO, in the presence of STO feeder cells (Materials and methods 2.7.3). The cells were then cultured for seven days before the total number of PGCs per well was counted. As in the previous experiments viable cells were distinguished from dead cells by the addition of trypan blue dye (Materials and methods 2.7.4) and the proportion of viable cells calculated to be 90% of cells in the control wells compared to approximately 10% of cells treated with the inhibitor. These results indicate that proliferation of the PGCs in the presence of inhibitor 420099, was significantly inhibited in comparison to proliferation under control conditions (Figure 4.5b). These results suggest that the phosphorylation of STAT3 and signalling through the JAK/STAT pathway is essential for the proliferation of chicken PGCs *in vitro*.

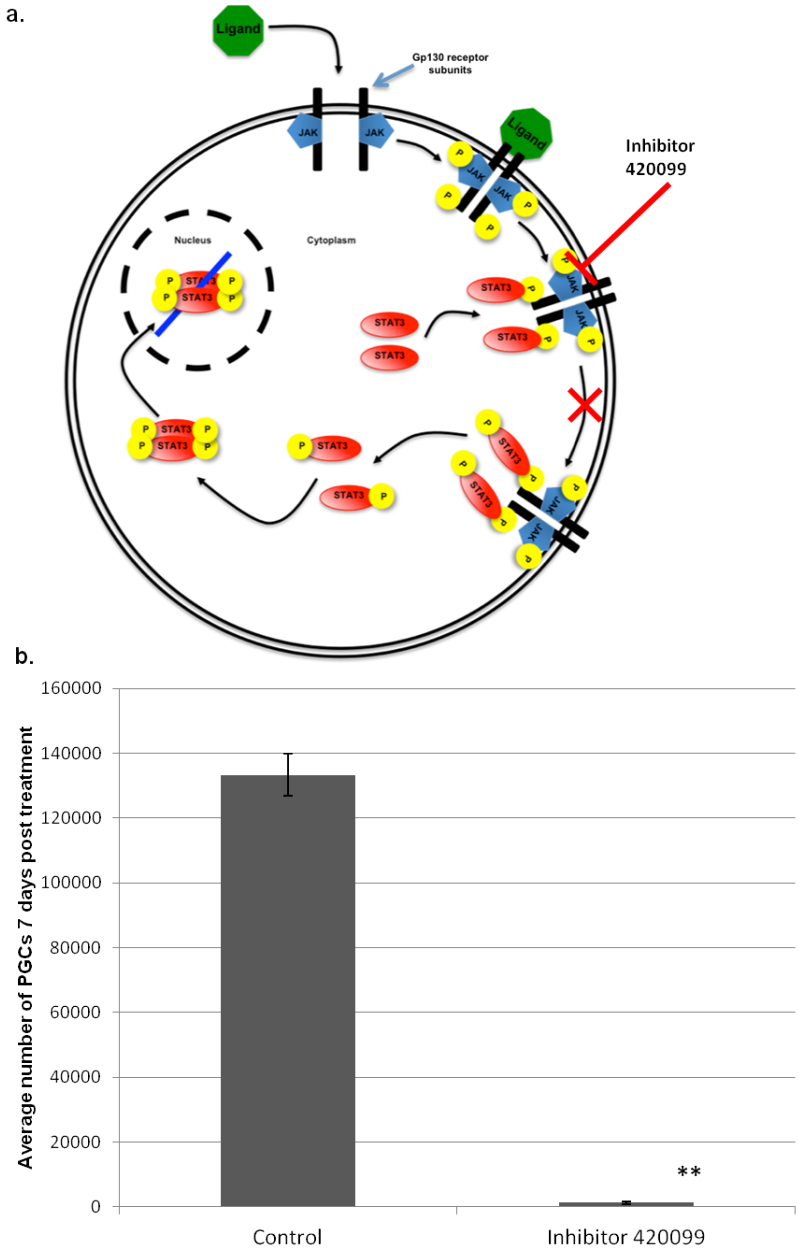


Figure 4.5 The effect of inhibitor 420099 on proliferation of PGCs *in vitro*. (a) Binding of the GP130 receptor by one of its ligands induces dimerisation of the transmembrane receptor and phosphorylation of the receptor associated protein JAK. Phosphorylation of JAK induces phosphorylation of the gp130 receptor that recruits STAT3. STAT3 becomes phosphorylated and dissociates from the receptor. By treating cells with the JAK inhibitor 420099, STAT3 does not become phosphorylated at the tyrosine residue 705 so cannot dissociate from the receptor, form dimers or translocate to the nucleus. This results in a deregulation of DNA transcription. Chicken PGCs from three lines, 08.08.09, 03.08.09 and 06.10.10 were treated with either DMSO (Control) or inhibitor 420099. Each cell line was assayed 3-6 times in three separate experiments. Wells were seeded with 1000 chicken PGCs 24 hrs prior to addition of the individual treatments. After 7 days trypan blue staining was used to assess cell viability. The average number of cells was calculated for each treatment group. Error bars, S.E.M. **, $P<0.01$

4.3.2 The effect of culture medium components on the activation of PI3K/AKT, MEK/ERK and JAK/STAT pathways in cultured chicken PGCs

The inhibitor experiments described in section 4.3.1 clearly demonstrated that signalling through each of the three pathways, PI3K/AKT, MEK/ERK and JAK/STAT, is essential for the proliferation of chicken PGCs *in vitro*. To identify specific components of the culture system that stimulate these pathways a series of induction experiments were carried out. Induction with each component was carried out in replicate and each sample was used for biochemical analysis of the three separate pathways.

4.3.2.1 Starvation and stimulation of chicken PGCs

To obtain protein for western blot analysis chicken PGCs from line 08.08.09 were grown in minimal medium (Materials and methods 2.1.6) for four hours, to induce a down-regulation of signalling pathways without compromising cell survival. After four hours components of the standard PGC culture medium were added individually to the starved cell: BRL conditioned medium, chicken and bovine sera, mouse and chicken SCF, human bFGF. Additionally the effects of human IGF1 and chicken, mouse and human LIF were also tested. To determine stimulation of the three pathways each factor was added at a range of concentrations and the cells were incubated for 15 min at 37^{o}C before the cell lyses was carried out using 1x SDS buffer to extract the cellular protein. The protein was then resolved on a 12% acrylamide gel, transferred to Hybond PVDF membrane and probed with antibodies specific to the individual pathways. An antibody that detects chicken gamma tubulin (Table 2.1) was used in all experiments to ensure that the amount of protein per sample was similar within experimental groups.

4.3.2.2 Analysis of phosphorylated AKT in PGCs cultured in the presence of individual components of the culture medium

As shown in section 4.3.1.2 signalling through the PI3K/AKT pathway is required for chicken PGC proliferation *in vitro*. To identify which of the individual components of the medium stimulated AKT phosphorylation proteins extracted from

the chicken PGCs and bound to a membrane were probed using an antibody that detects phosphorylated AKT (Table 2.1). The antibody, phosphAKT (Ser473) (Cell Signalling) binds to AKT phosphorylated at serine residue 473. As shown in figure 4.2 this residue is conserved between several species; chicken, mouse, rat, bovine and human. Pan AKT antibody (Table 2.1) was used to show the presence of total protein in each sample. AKT was unphosphorylated in all the starved chicken PGC control samples and phosphorylated under standard culture conditions (basic medium plus hFGF2).

4.3.2.2.1 Analysis of AKT phosphorylation in PGCs cultured in the presence of SCF

SCF, an activator the PI3K/AKT pathway was obtained from chicken and mouse. Each SCF was added to the starved chicken PGC cultures at different concentration, chicken SCF (cSCF) at 1U/ml and 10U/ml and the mouse SCF (mSCF) at 15, 25 and 50ng/ml. The results (Figure 4.6a) show that AKT phosphorylation is induced in the chicken PGCs that were stimulated with cSCF at a concentration of 10U/ml. In contrast cells treated with cSCF at 1U/ml or mSCF, at any of the concentrations tested did not induce phosphorylation of AKT. Analysis of the amino acid sequences shows that the SCF is 56% conserved between chicken and mouse (Figure 4.7) but functional conservation is unknown.

4.3.2.2.2 Analysis of AKT phosphorylation in PGCs cultured in the presence of IGF1

IGF1 is not a component of the van de Lavoir culture medium but has been shown to be required for PGC survival, migration and colonisation of the gonad in zebrafish (Schlueter, Peng, *et al*. 2007; Schlueter, Sang, *et al*. 2007). IGF1 has also been suggested to have a positive effect on chicken PGC culture (Wang and Du, 2004). When signalling through IGF1 is suppressed it results in a reduction in AKT phosphorylation implicating it as a key inducer of the PI3K/AKT pathway (Sang *et al*. 2008). The chicken IGF1 amino acid sequence is 66% identical to mouse, 79% identical to rat and 83% conserved with human and bovine as demonstrated by

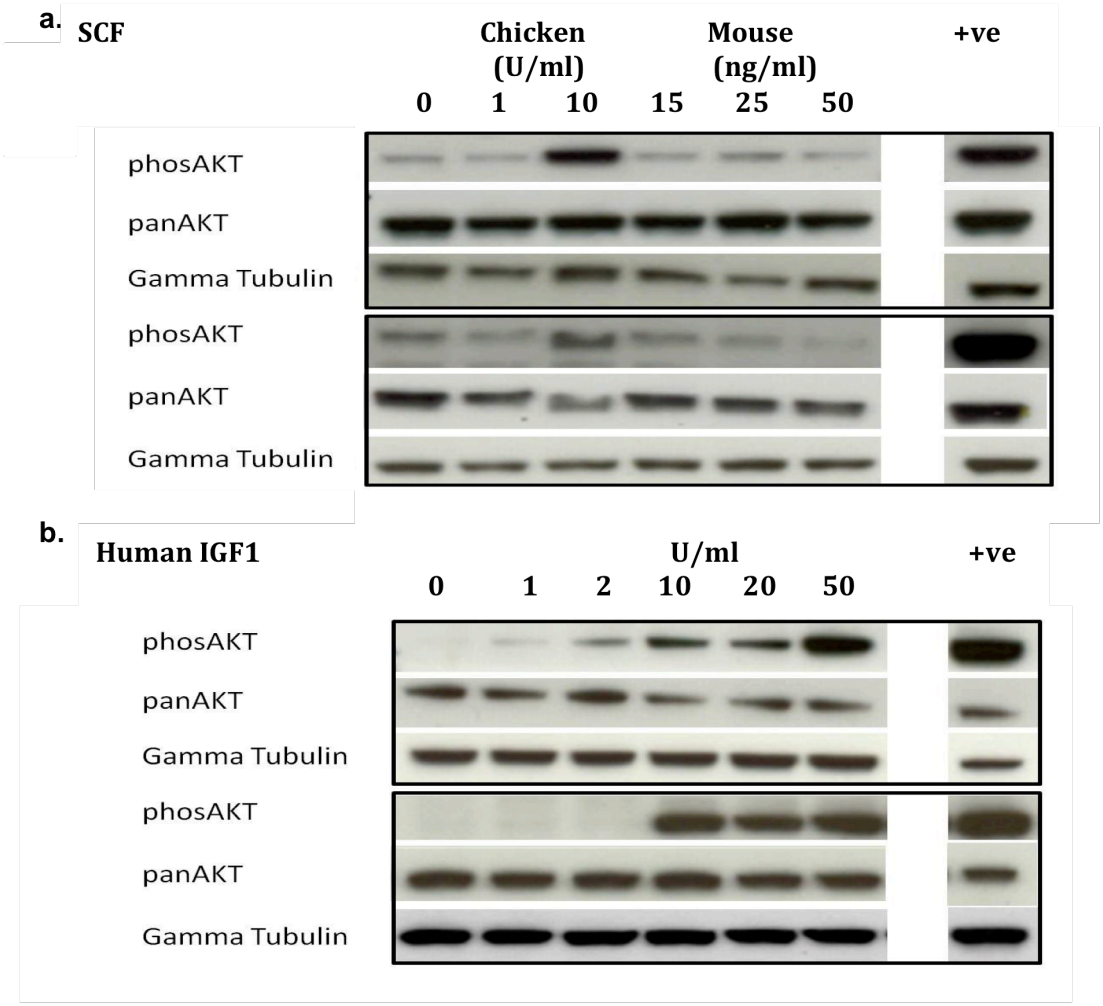


Figure 4.6 Analysis of AKT expression in PGCs cultured in the presence of SCF or IGF1. Western analysis of total protein from chicken PGCs line 08.08.09, cultured in starvation medium for 4 hours and then in the presence of (a) chicken SCF at (1 and 10 U/ml) and mouse SCF (15, 25 and 50ng/ml) (b) hIGF1 (1-50 U/ml) for 15 minutes. The protein was probed for phosphorylated AKT. AKT is phosphorylated under standard culture (Basic medium plus hFGF2) conditions (+ve). All samples were positive for total AKT protein (panAKT). Gamma tubulin shows that the protein was similar between samples. Experiments were carried out in replicated as represented by the individual panels.

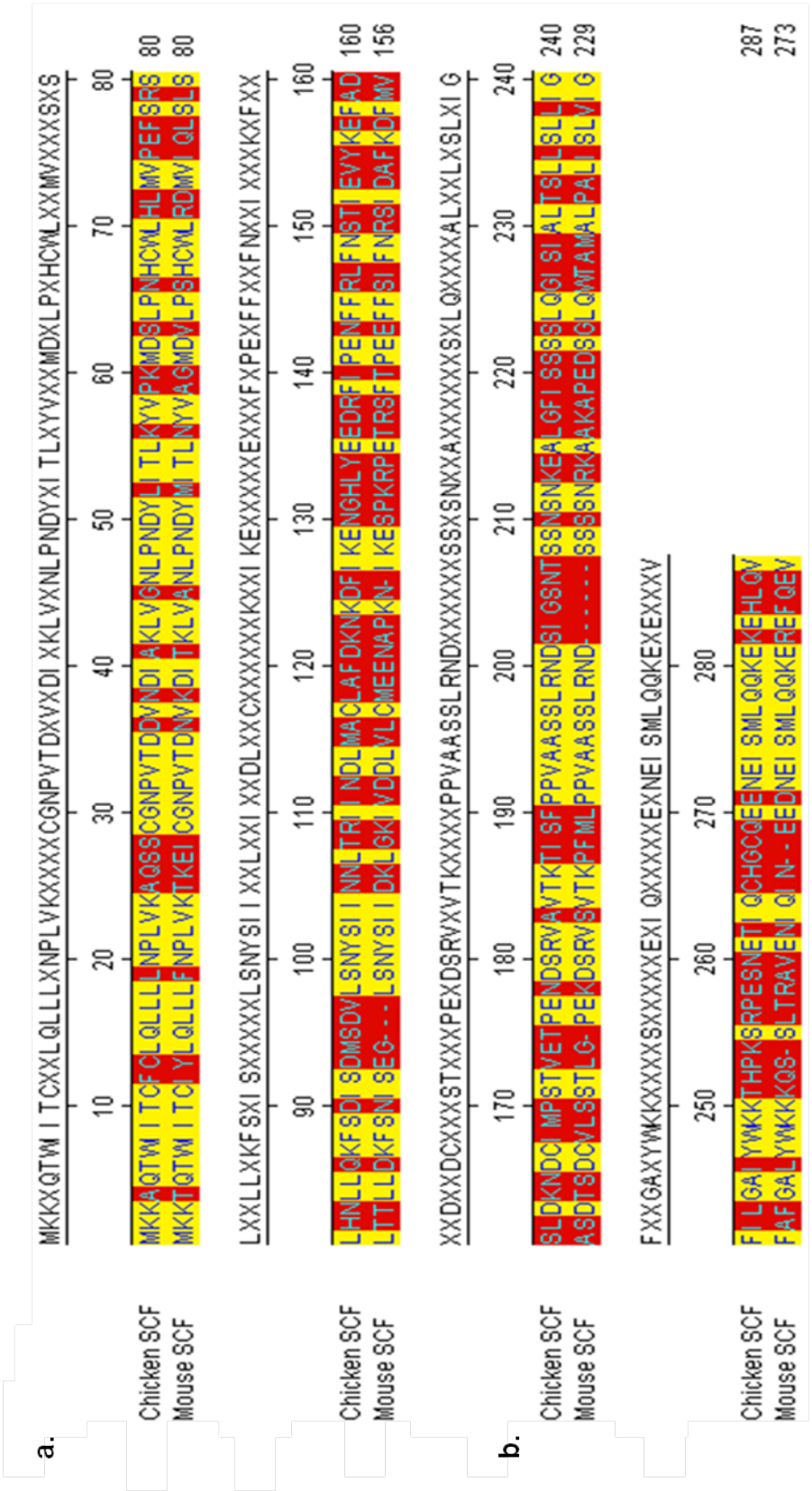


Figure 4.7 Comparison of SCF sequence in chicken and mouse. Alignment of the mouse and chicken SCF polypeptides shows 56% sequence identity between the species. Conserved residues are highlighted in yellow.

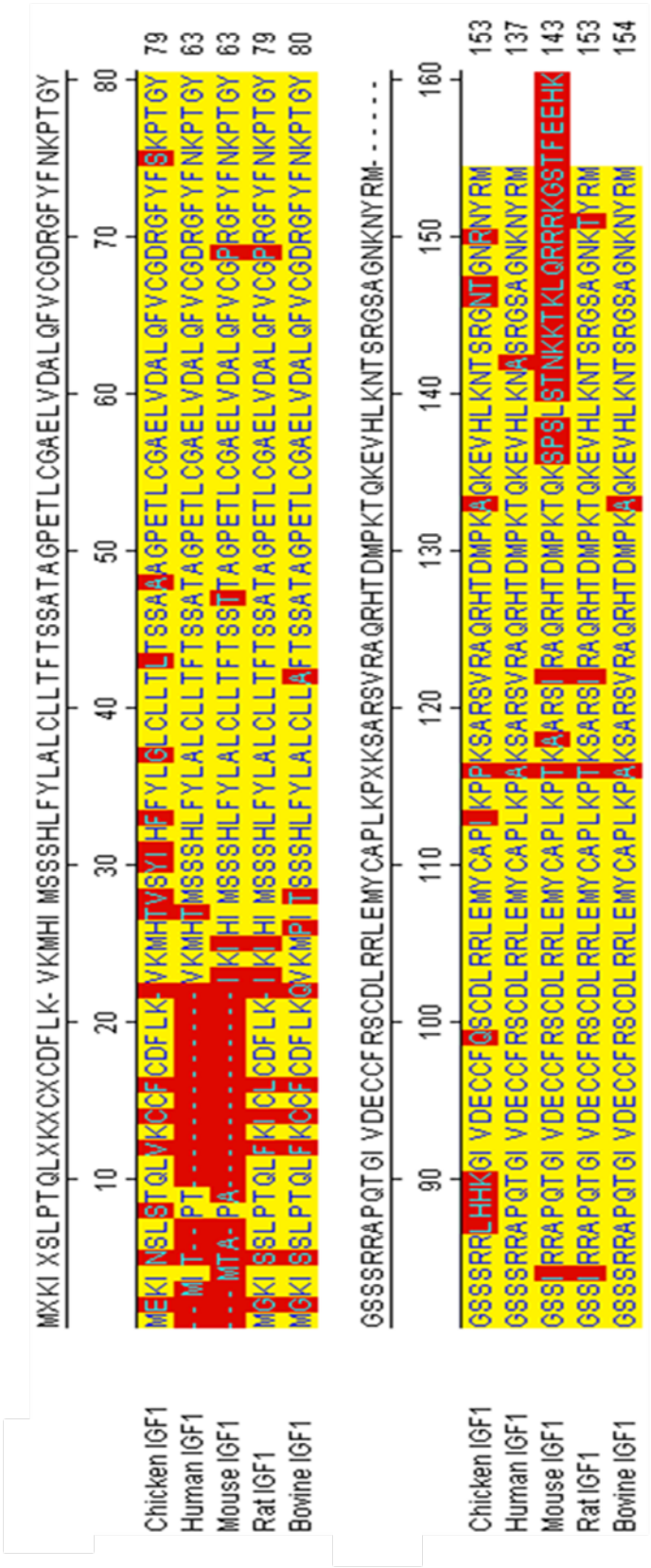


Figure 4.8 Comparison of IGF1 sequence in vertebrates. Alignment of the IGF1 sequences from chicken, human, mouse, rat and bovine show that IGF1 is 66 to 83% similar between the five species. Conserved residues are highlighted in yellow.

alignment of the amino acid sequences (Figure 4.8d). Functional conservation of IGF1 in chicken cell culture may vary depending on the vertebrate species. Human IGF1 (hIGF1) was added to the starved PGCs at concentrations increasing from 1 to 50U/ml and the cellular protein extracts probed for the presence of phosphorylated AKT. Phosphorylated AKT was detected in experimental replicates of protein from PGCs treated with hIGF1 added at concentrations of 10U/ml or more (Figure 4.6b). From the results of the first experiment (Figure 4.6b, top panel) phosphorylation of AKT was detected when the hIGF1 was added at concentrations of 1 and 2U/ml and that as hIGF1 increased, the amount of phosphorylated AKT protein increased. These results indicated that hIGF1 was able to induce the PI3K/AKT pathway. Therefore IGF1 expressed by BRL and STO cells may cause activation of the pathway in chicken PGCs *in vitro*.

4.3.2.2.3 Analysis of AKT phosphorylation in PGCs cultured in the presence of BRL-conditioned medium

BRL-conditioned medium (Materials and methods 2.1.6) contributes to 50% of the van de Lavoir culture medium. BRL cells are known to produce LIF and IGF1 and are likely to secrete a variety of other growth factors. FBS, which is added to the conditioned medium, contains growth factors and cytokines at unknown concentrations, which could contribute to induction of the cellular signalling pathways investigated here. Conditioned medium, at concentrations increasing from 2.5% to 50%, was added to the PGCs grown in minimal medium and the total cellular protein extracted. The protein sample was then probed for AKT phosphorylated at serine residue 473 (Figure 4.9). The western blot analysis showed that addition of BRL-conditioned medium to the cells in culture induced AKT phosphorylation. The amount of phosphorylated AKT did not increase with increased amounts of BRL-conditioned medium. This suggests that the lowest concentration of BRL-conditioned medium was efficient to fully induce AKT phosphorylation. The factor present in the BRL-conditioned medium that induced the activation is unlikely to be SCF. Any SCF would be from the FBS or secreted from the BRL cells and alignment of the amino acid sequences show that chicken SCF has

53% sequence identity with SCF from rat (Figure 4.10a) and bovine (Figure 4.10b). These results suggest that these sequences are likely not to be functionally conserved with the chicken SCF. However IGF1, which is expressed by the BRL cells and may be functionally conserved between the vertebrate species, may be the factor responsible of inducing activation of AKT.

4.3.2.1.4 Analysis of AKT phosphorylation in PGCs cultured in the presence of sera

Two animal sera are used in the culture of chicken PGCs, chicken serum and FBS. Sera contain unknown amounts of growth factors and cytokines and have been shown to promote cell survival in culture. Chicken sera and FBS are added to chicken PGC culture medium at 2.5 and 7.5% respectively. Both were assessed for their ability to induce phosphorylation of AKT in culture chicken PGCs. Each sera was added to starved chicken PGC cultures and the total cellular protein extracted probed for phosphorylated AKT. The starved cells were treated with chicken serum or FBS at amounts increasing from 2.5% to 50%. Interestingly no induction of AKT was observed in any of the samples for either of the sera (Figure 4.11 a and b).

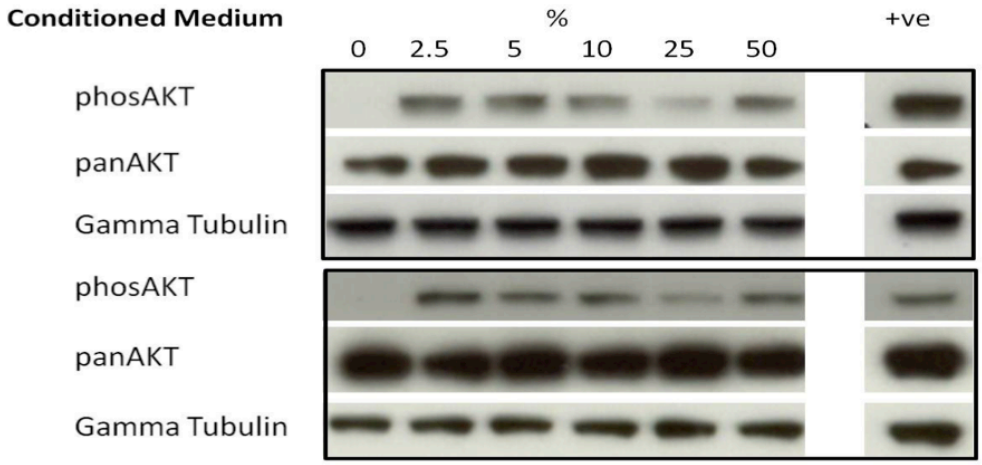


Figure 4.9 Analysis of AKT expression in PGCs cultured in the presence of BRL-conditioned medium. Western analysis of total protein from chicken PGCs line 08.08.09, cultured in starvation medium for 4 hours and then in the presence of BRL-conditioned medium (0, 2.5, 5, 10, 25, 50%). for 15 minutes. The protein was probed for phosphorylated AKT. AKT is phosphorylated under standard culture (Basic medium plus hFGF2) conditions (+ve). All samples were positive for total AKT protein (panAKT). Gamma tubulin shows that the protein was similar between samples. Experiments were carried out in replicated as represented by the individual panels.

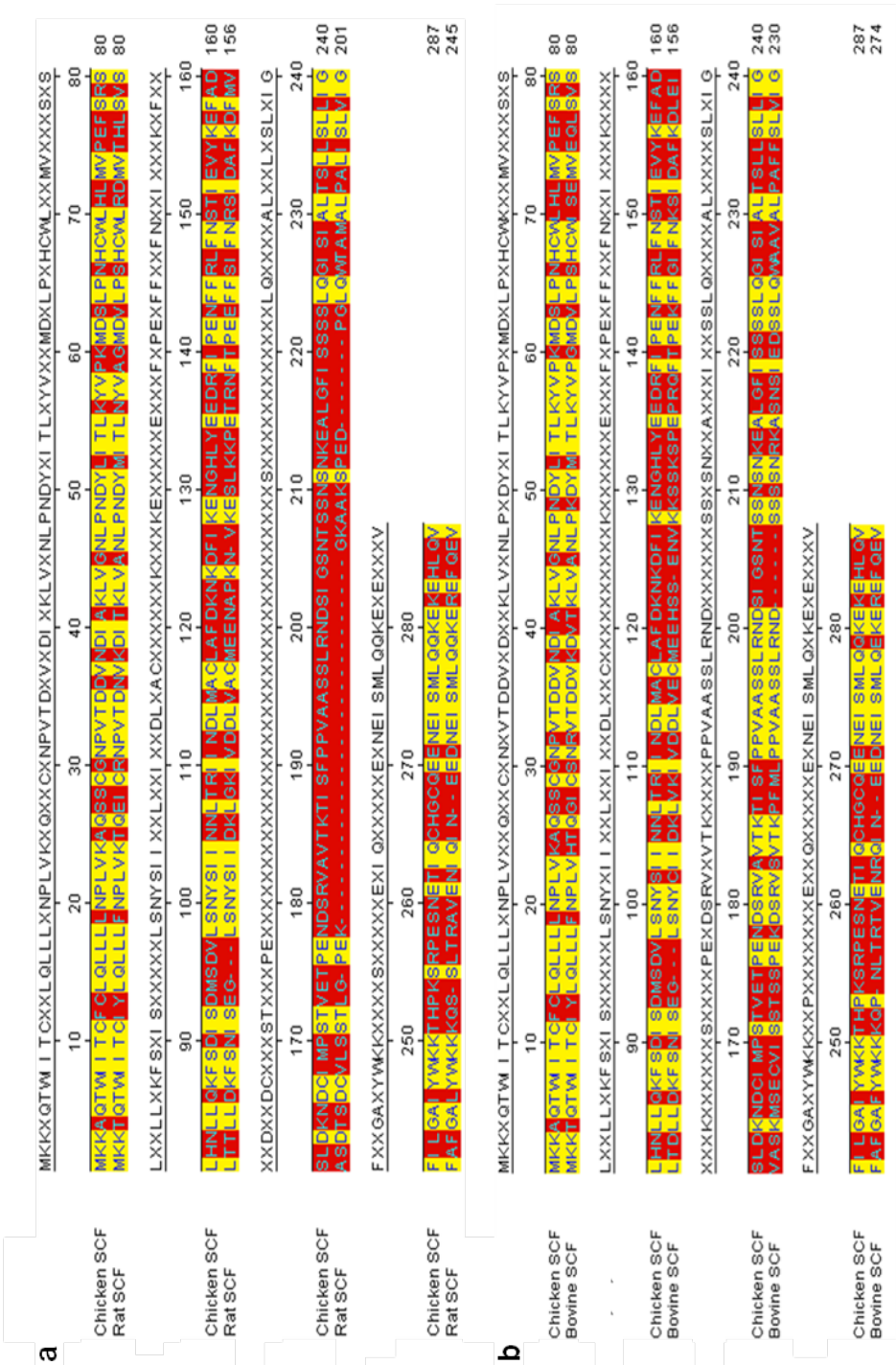


Figure 4.10 Comparison of SCF sequences in chicken, rat and bovine. Alignment of the chicken SCF sequence with (a) rat and (b) bovine. The SCF polypeptides show 53% sequence identity between the species. Conserved residues are highlighted in yellow.

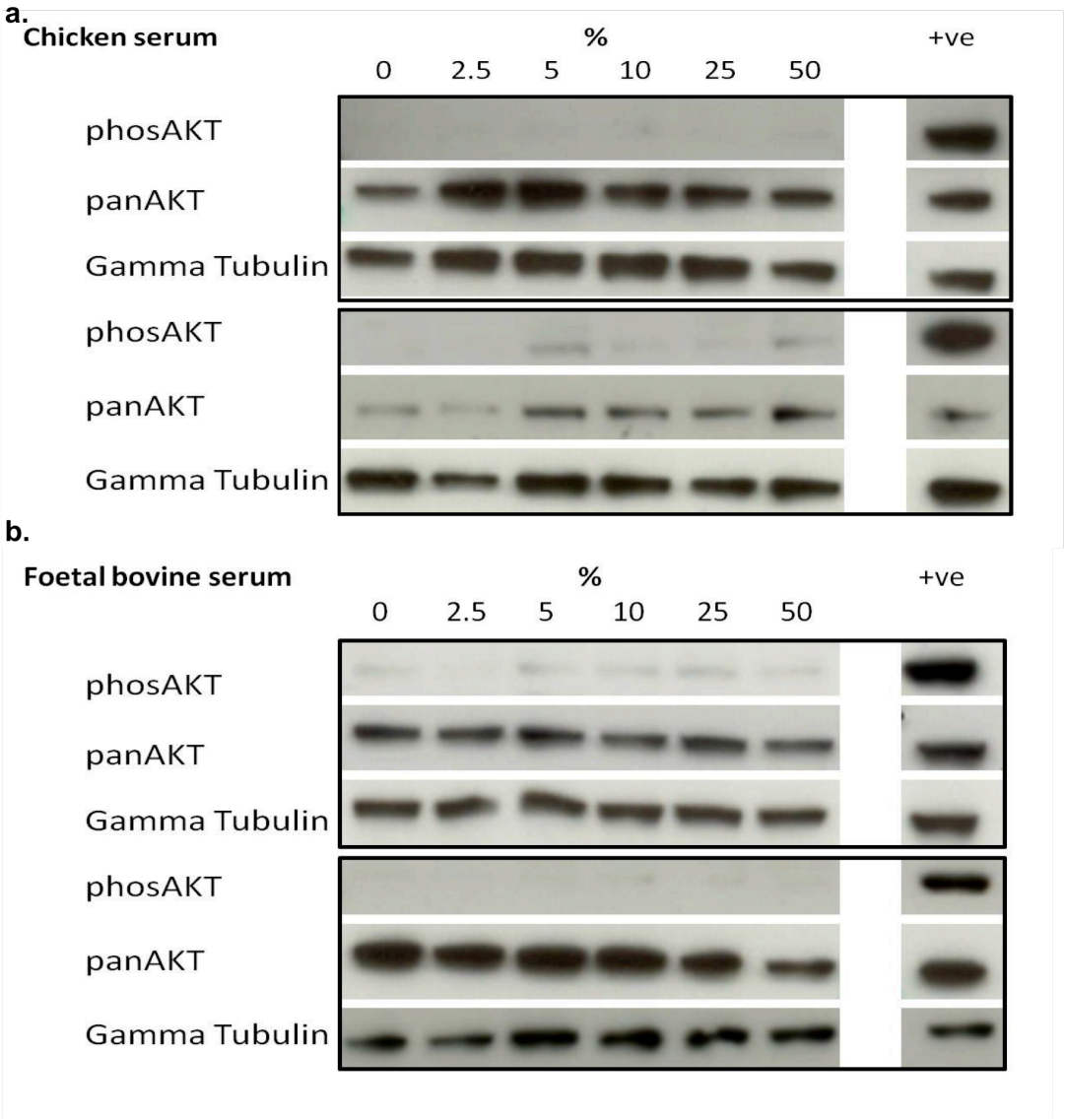


Figure 4.11 Analysis of AKT expression in PGCs cultured in the presence of sera. Western analysis of total protein from chicken PGCs line 08.08.09, cultured in starvation medium for 4 hours and then in the presence of (a) Chicken serum (0, 2.5, 5, 10, 25, 50%), (b) FBS (0, 2.5, 5, 10, 25, 50%) for 15 minutes. The protein was probed for phosphorylated AKT. AKT is phosphorylated under standard culture (Basic medium plus hFGF2) conditions (+ve). All samples were positive for total AKT protein (panAKT). Gamma tubulin shows that the protein was similar between samples. Experiments were carried out in replicated as represented by the individual panels.

4.3.2.1.5 Analysis of AKT phosphorylation in PGCs cultured in the presence of FGF2 or LIF

hFGF2 is added to culture medium as a purified factor whilst LIF is present in the BRL-conditioned medium and expressed by the STO feeder cells. To assess whether either of these factors induces the PI3K/AKT pathway in chicken PGCs, AKT phosphorylation was assessed. Total cellular protein extract from starved PGCS that had been cultured in the presence of hFGF2, chicken LIF (cLIF), mouse LIF, (mLIF) or human LIF (hLIF) were probed for phosphorylated AKT. Phosphorylated AKT was not detected in any of the hFGF2 (Figure 4.12a) nor the LIF (Figure 4.12b) treated samples. This result was unsurprising, as neither FGF2 nor LIF have been reported to be activators of the AKT pathway.

4.3.2.3 The effect of culture medium components on MEK/ERK pathway activation

When the inhibitor PD0325901 was used to block signalling through the MEK/ERK proliferation of chicken PGCs *in vitro* was significantly inhibited (section 4.3.1.3). To identify which of the individual components of the culture medium stimulate the MEK/ERK pathway the protein extracted from chicken PGCs (Section 4.3.2.1) after induction with individual components of the medium was probed using the antibody phospho-44/42 MAPK (Erk1/Erk2) (Table 2.1). This antibody detects ERK1/2 phosphorylation at threonine residue 202 and tyrosine residue 204. As shown in section 4.3.1 and figures 4.1 4.2 and 4.4 this antibody can be used to detect the phosphorylated protein in chicken samples. An antibody that detects unphosphorylated ERK2; panERK (Table 2.1) was used to show the presence of total protein in each sample. ERK1/2 was unphosphorylated in all starved PGC control samples and phosphorylated under standard culture conditions (basic medium plus hFGF2).

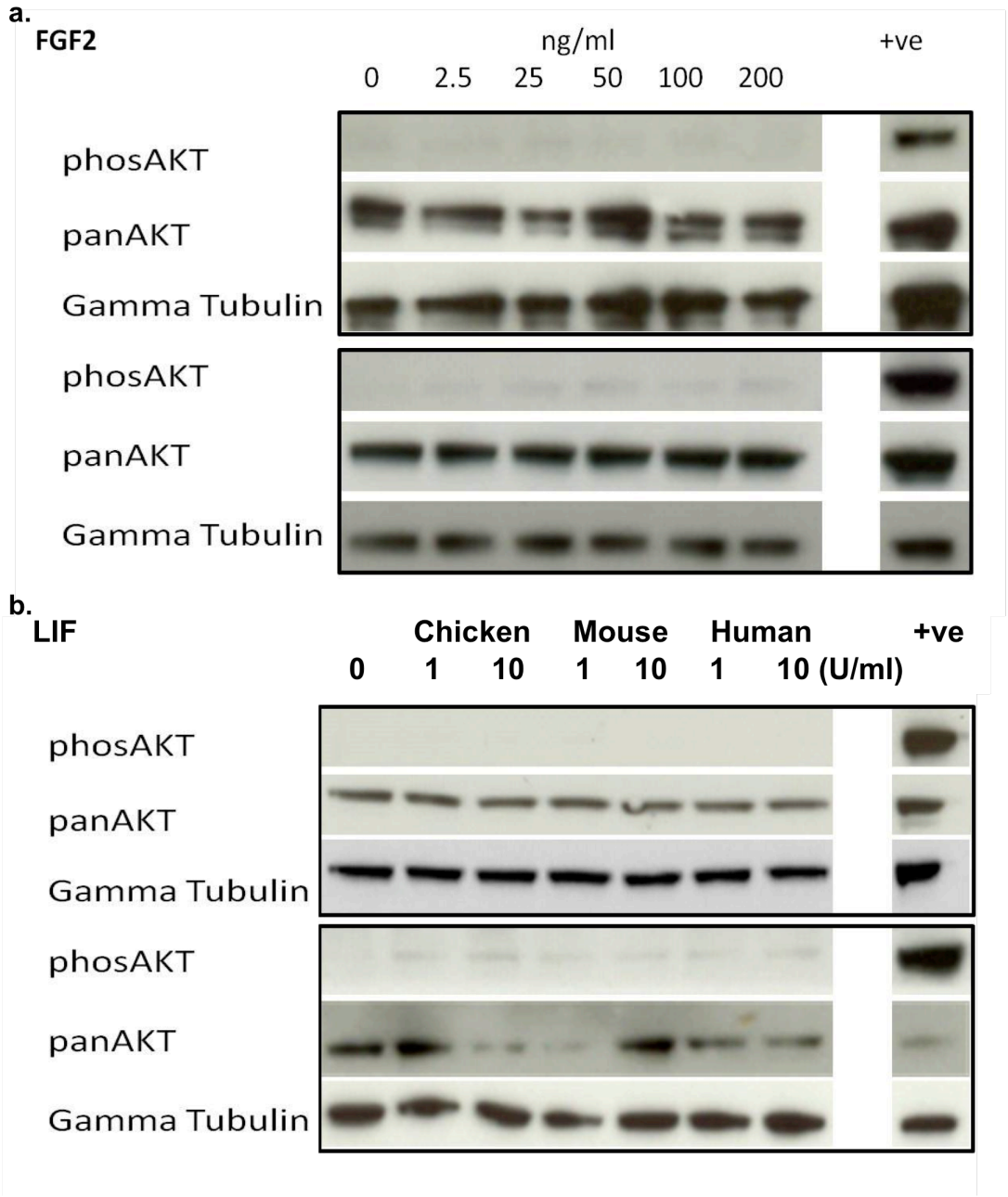


Figure 4.12 Analysis of AKT expression in PGCs cultured in the presence of hFGF2 or LIF. Western analysis of total protein from chicken PGCs line 08.08.09, cultured in starvation medium for 4 hours and then in the presence of (a) hFGF2 (2.5, 25, 50, 100 and 200ng/ml) and (b) chicken, mouse and human LIF (1 and 10U/ml) for 15 minutes. The protein was probed for phosphorylated AKT. AKT is phosphorylated under standard culture (Basic medium plus hFGF2) conditions (+ve). All samples were positive for total AKT protein (panAKT). Gamma tubulin shows that the protein was similar between samples. Experiments were carried out in replicated as represented by the individual panels.

4.3.2.3.1 Analysis of ERK1/2 phosphorylation in PGCs cultured in the presence of FGF2

FGF2 can bind FGF receptors and induce MEK/ERK pathway activation in mouse and chicken (Böttcher and Niehrs 2005; Choi *et al*. 2010). Alignments of the amino acid sequences show that the FGF2 protein is 90% conserved between several vertebrate species, chicken, rat, bovine, mouse and human (Figure 4.13) and therefore FGF2 from different vertebrates is likely to be functional in chicken cells. To identify if hFGF2 that is added to culture medium induces the MEK/ERK pathway in the chicken PGCs the hFGF2 induced total proteins used in section 4.3.2.1.5 were probed for phosphorylated ERK1/2. Phosphorylated ERK1/2 was identified in all the samples of both replicates with the exception of one (Figure 4.14a). It is predicted that this was likely to be the result of a pipetting error. All the other results show a clear induction of ERK1/2 phosphorylation. The level of induction did not increase with increasing concentration of the growth factor indicating that at 2.5ng/ml of hFGF2 is enough to fully activate the MEK/ERK pathway in the tested chicken PGCs.

4.3.2.3.2 Analysis of ERK1/2 phosphorylation in PGCs cultured in the presence of BRL- conditioned medium

BRL-conditioned medium contains several growth factors that are secreted from the BRL cells during the production process one of which is LIF, an activator of MEK/ERK pathway (Figure 1.7). The total protein extracted from chicken PGCs treated with BRL-conditioned medium (section 4.3.2.1) was assayed for MEK/ERK activation by probing for phosphorylated ERK1/2. Increasing amounts of phosphorylated ERK1/2 were detected with the addition of increasing concentrations of BRL-conditioned medium (Figure 4.14b). However alignment of the chicken LIF amino acid sequences with the sequences of other vertebrates: rat, mouse, human and bovine show a low level of sequence conservation (Figure 4.15). Horiuchi *et al*. (2004) demonstrated that chicken ES cells cultured in the presence of mouse LIF are unable to maintain an undifferentiated state whilst chicken LIF inhibits

differentiation. This indicated that mouse and chicken LIF do not retain functional conservation.

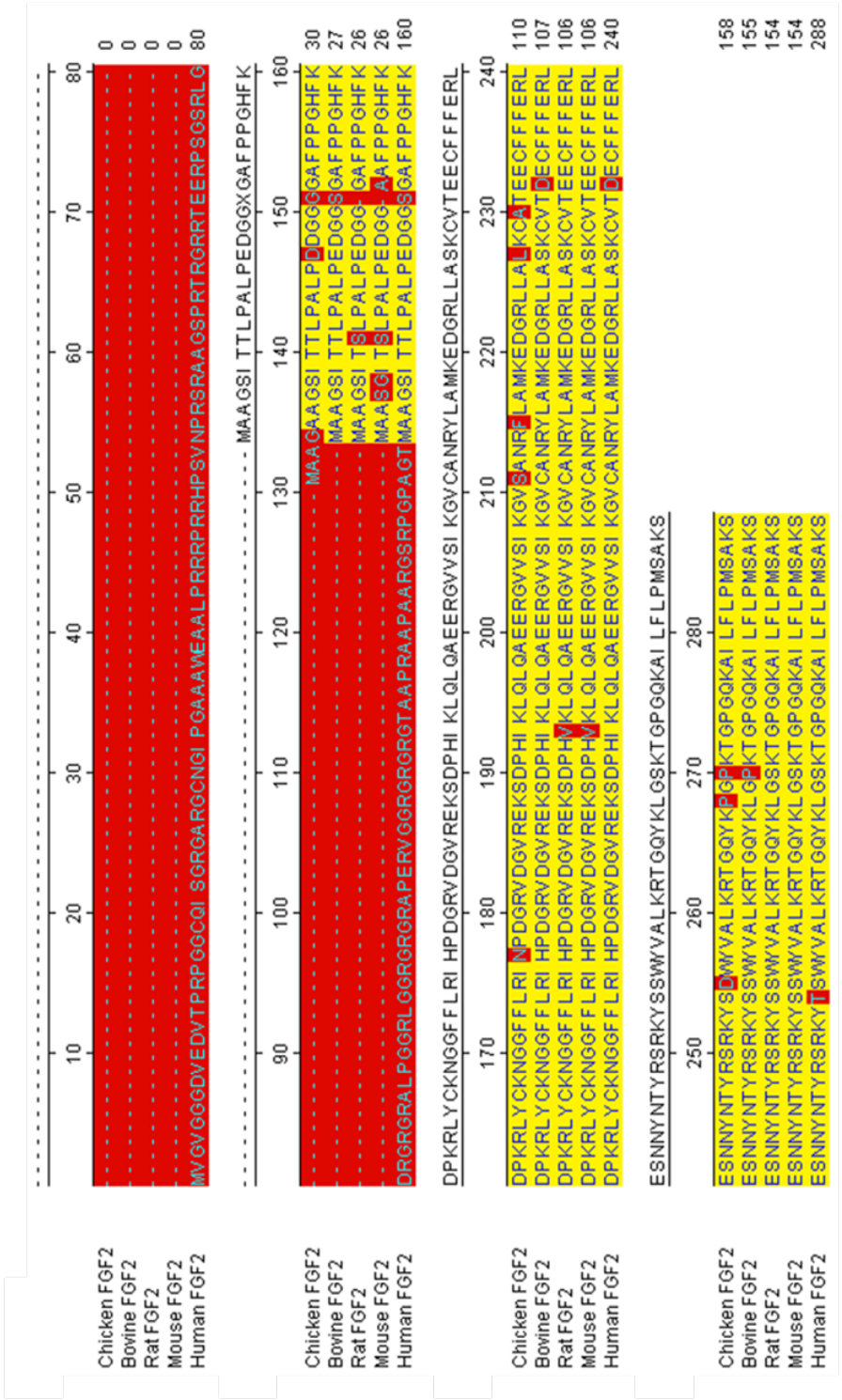


Figure 4.13 Comparison of vertebrate FGF2 sequences. Alignment of the FGF2 sequences from chicken, human, mouse, rat and bovine show that FGF2 is over 90% similar between species. Conserved residues are highlighted in yellow.

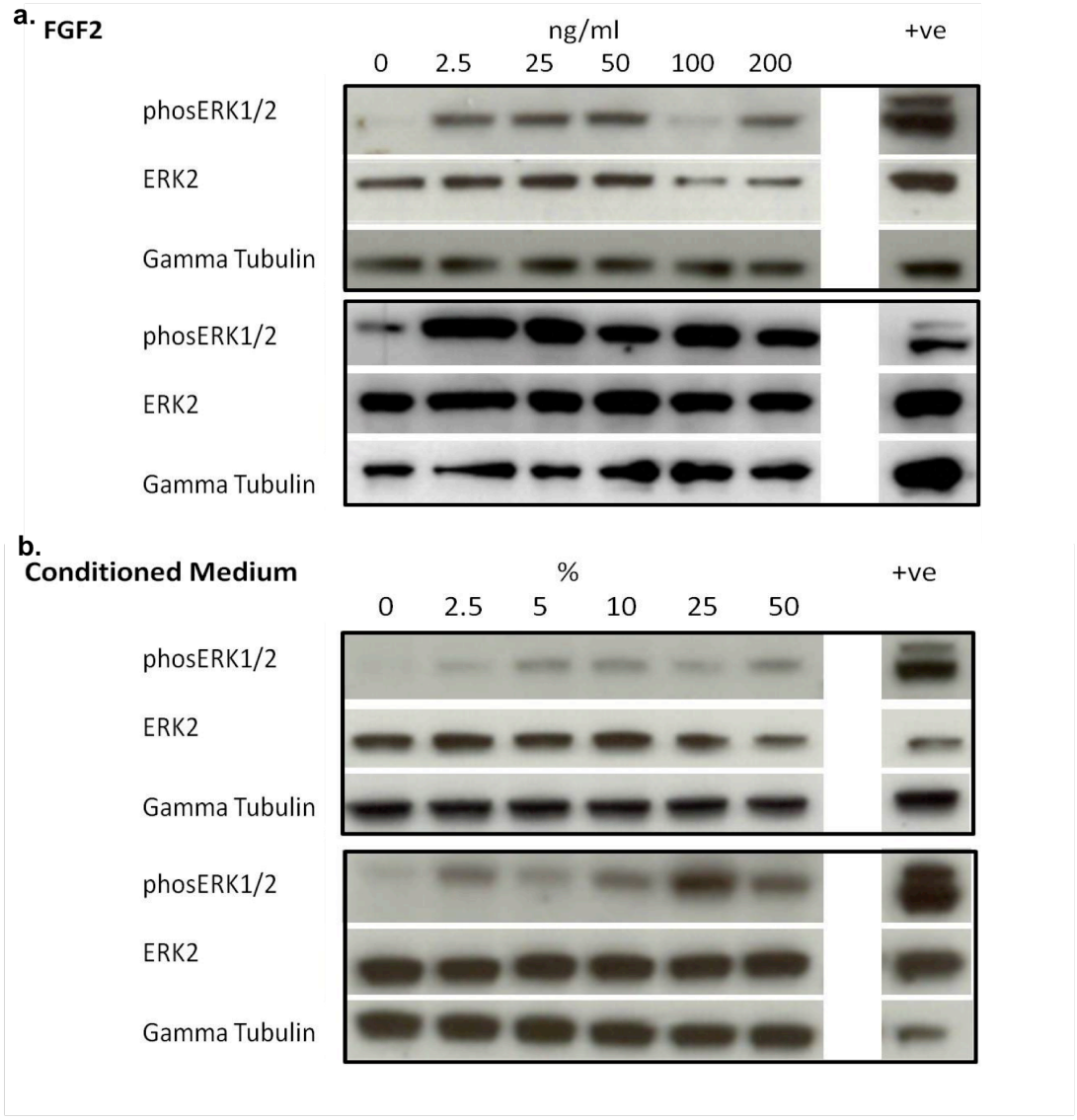
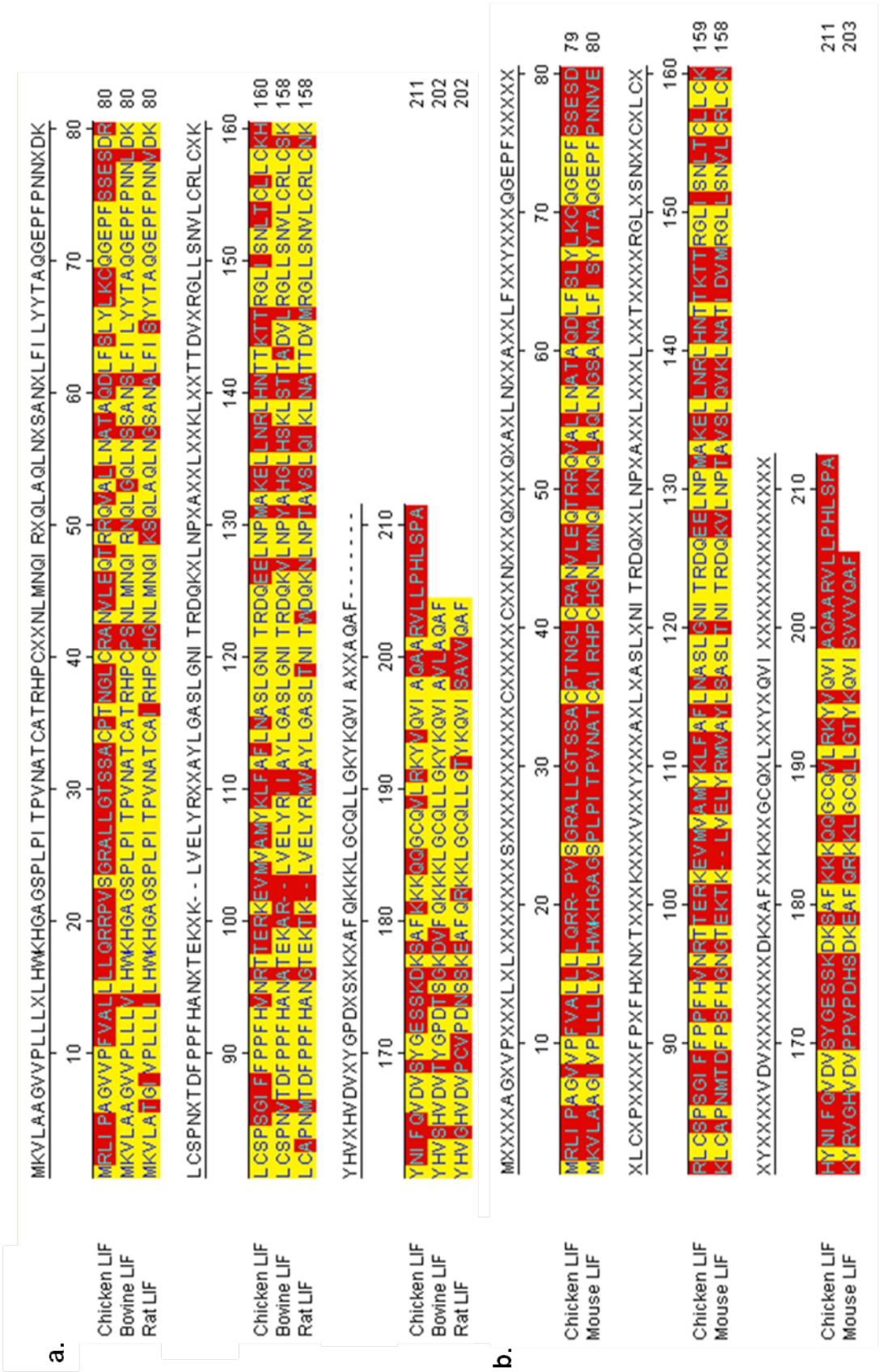


Figure 4.14 Analysis of ERK1/2 expression in PGCs cultured in the presence of hFGF2 and BRL-conditioned medium. Western analysis of total protein from chicken PGCs line 08.08.09, cultured in starvation medium for 4 hours and then in the presence of (a) hFGF2 (2.5, 25, 50, 100 and 200ng/ml), (b) BRL-conditioned medium (0, 2.5, 5, 10, 25, 50%) for 15 minutes. The protein was probed for phosphorylated ERK1/2. ERK1/2 is phosphorylated under standard culture (Basic medium plus hFGF2) conditions (+ve). All samples were positive for total ERK2 protein (ERK2). Gamma tubulin shows that the protein was similar between samples. Experiments were carried out in replicated as represented by the individual panels.


a.
Chicken LIF
Bovine LIF
Rat LIF
b.
Chicken LIF
Mouse LIF

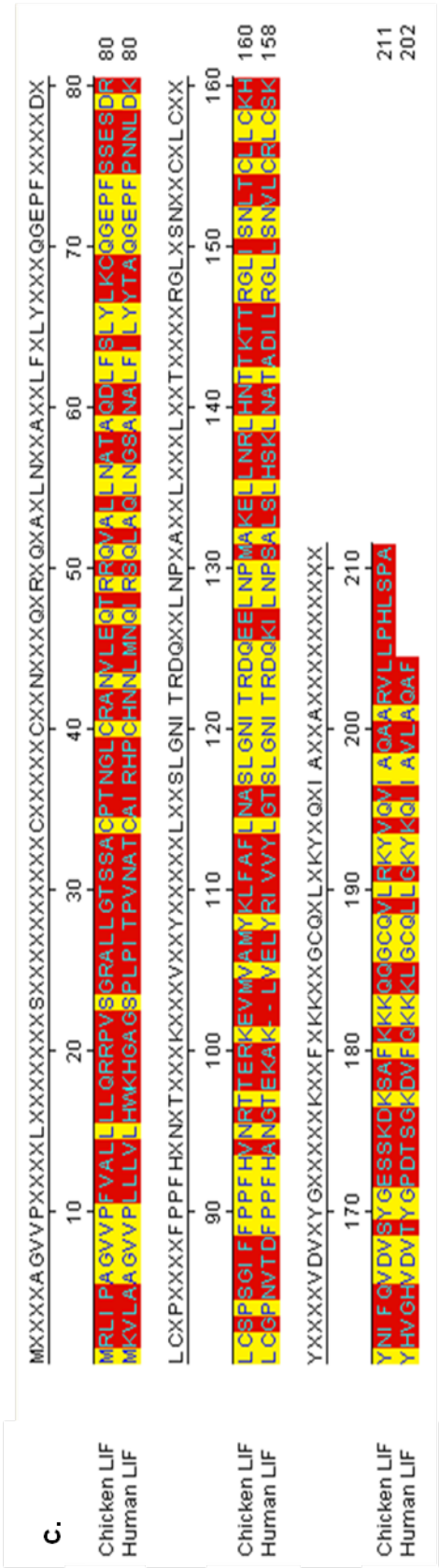


Figure 4.15 Comparison of vertebrate LIF sequences. (a) The alignment of the LIF sequence from chicken, bovine and rat show that chicken LIF is 44% similar to bovine and 36% similar to rat. (b) Sequence analysis of chicken and mouse LIF shows they are 39% similar. (c.) The chicken and human LIF sequences have 47% similarity. Conserved residues are highlighted in yellow.

The amino acid sequence of mouse and rat LIF are almost 100% conserved. This indicates that LIF secreted from the BRL cells is unlikely to activate the MEK/ERK pathway in the chicken PGCs. It is likely that another factor present in the BRL-conditioned medium, such as IGF1 or a component of the FBS is responsible for the observed induction of the pathway in the starved chicken PGCs. As mentioned in section 4.3.2.1.2, IGF1 is conserved between species and therefore a suitable candidate for the induced ERK1/2 phosphorylation detected in figure 4.14b.

4.3.2.3.3 Analysis of ERK1/2 phosphorylation in PGCs cultured in the presence of sera from chicken and bovine

Both FBS and chicken sera contain unknown amounts of undefined growth factors likely to cause induction of signalling pathways such as MEK/ERK. The protein extracted from chicken PGCs treated with either chicken serum or FBS (section 43214) were analysed for induction of ERK1/2 phosphorylation. Phosphorylated ERK1/2 was detected in both the chicken sera (Figure 4.16a) and the FBS (Figure 4.16b) treated protein samples. ERK1/2 phosphorylation was similar in all the chicken sera treated samples. This indicated that even at the lowest level used, 2.5%, chicken sera was sufficient to induce maximal activation of the MEK/ERK pathway in the starved chicken PGCs. In contrast the level of ERK1/2 phosphorylation resulting from FBS stimulation increased with increasing concentration. This was seen clearly in experiment one but not experiment 2 (Figure 4.16b). These results indicated the induction of ERK1/2 phosphorylation observed in the BRL-conditioned medium induction might have been induced by the FBS.

4.3.2.3.4 Analysis of ERK1/2 phosphorylation in PGCs cultured in the presence of LIF or IGF1

As mentioned previously LIF can activate the MEK/ERK pathway by binding the LIFR/GP130R receptor dimer (Figure 1.9). Despite the likely lack of functional conservation LIF from different vertebrates in chicken cells (Section 4.3.2.2.2; Figure 4.15) the effects of three LIF proteins were assessed for induction of ERK1/2 phosphorylation in the starved chicken PGCs. Total protein extract from the chicken

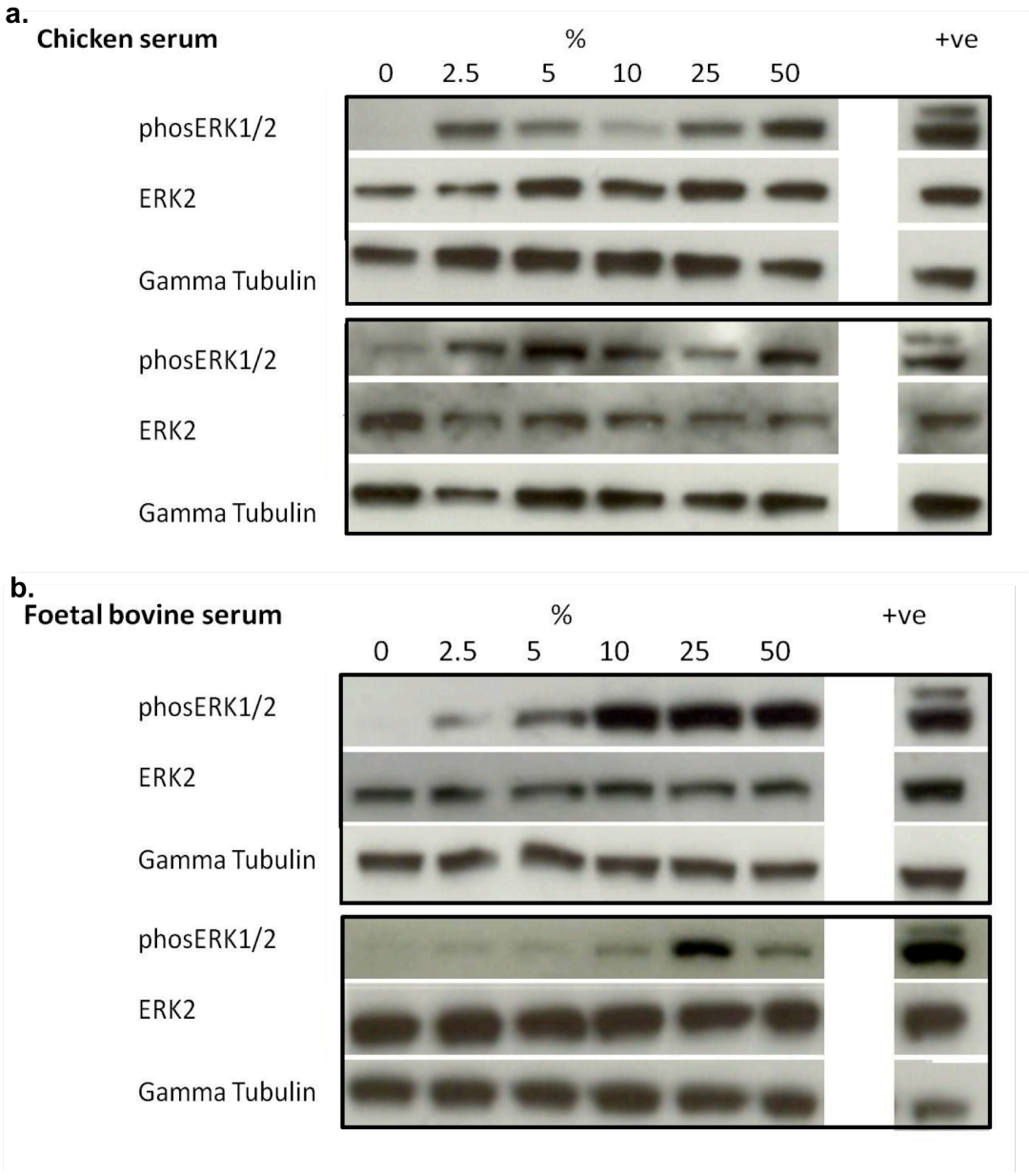


Figure 4.16 Analysis of ERK1/2 expression in PGCs cultured in the presence of sera. Western analysis of total protein from chicken PGCs line 08.08.09, cultured in starvation medium for 4 hours and then in the presence of (a) Chicken serum (0, 2.5, 5, 10, 25, 50%), (b) FBS (0, 2.5, 5, 10, 25, 50%) for 15 minutes. The protein was probed for phosphorylated ERK1/2. ERK1/2 is phosphorylated under standard culture (Basic medium plus hFGF2) conditions (+ve). All samples were positive for total ERK2 protein (ERK2). Gamma tubulin shows that the protein was similar between samples. Experiments were carried out in replicated as represented by the individual panels.

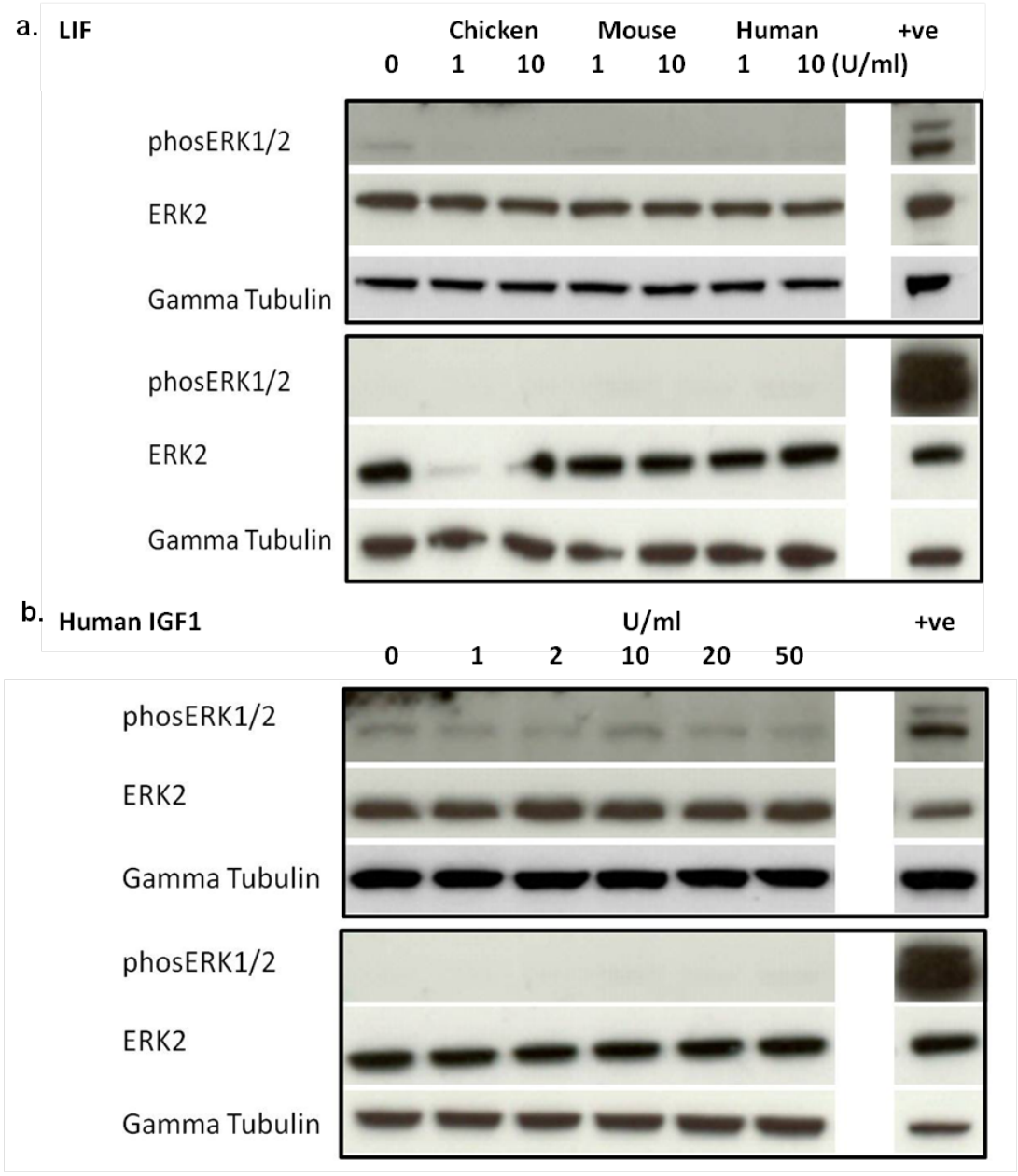


Figure 4.17 Analysis of ERK1/2 expression in PGCs cultured in the presence of LIF or hIGF1. Western analysis of total protein from chicken PGCs line 08.08.09, cultured in starvation medium for 4 hours and then in the presence of (a) chicken, mouse and human LIF (1 and 10U/ml) (b) hIGF1 (100-5000U/ml), for 15 minutes. The protein was probed for phosphorylated ERK1/2. ERK1/2 is phosphorylated under standard culture (Basic medium plus hFGF2) conditions (+ve). All samples were positive for total ERK2 protein (ERK2). Gamma tubulin shows that the protein was similar between samples. Experiments were carried out in replicated as represented by the individual panels.

PGCs treated with either chicken, mouse or human LIF were probed for phosphorylated EER1/2. Figure 4.17a shows that no phosphorylated Erk1/2 was detected in any of the samples regardless of which species the LIF was from. Probing for total ERK2 protein confirmed that unphosphorylated protein was present in all the samples.

Unlike LIF, IGF1 is not known to induce signalling through the MEK/ERK pathway but as demonstrated previously (Section 4.3.2.1.3; Figure 4.8) IGF1 is conserved between species and hIGF1 is functional in chicken PGCs as demonstrated by induction of AKT phosphorylation (Figure 4.6b). Total protein extracted from starved chicken PGCs treated with hIGF1 was assessed for phosphorylated ERK1/2. As expected figure 4.17 shows that ERK1/2 phosphorylation was not induced in chicken PGCs treated with hIGF1. Therefore any IGF1 present in the BRL-conditioned medium is not inducing the activation of this pathway in chicken PGCs cultured *in vitro.*

4.3.2.3.5 Analysis of ERK1/2 phosphorylation in PGCs cultured in the presence of SCF

SCF can activate the MEK/ERK pathway via a MEK dependent pathway (Dolci *et al et al*. 2001, Kapur *et al*. 2011). Western blot analysis of total protein extracted from the chicken PGCs that had been induced with SCF from chicken and mouse were probed for phosphorylated ERK1/2. Figure 4.18 shows detection of phosphorylated ERK1/2 in the protein lysates from the cells that were treated with chicken SCF at a concentration of 10U/ml in both experiment 1 and experiment 2. These were the only samples in which phosphorylated ERK1/2 was detected adding further weight to the conclusions made in section 4.3.2.1.1 and chapter 3 that mouse SCF is not functional in chicken PGCs. Detection of ERK2 total protein shows that ERK2 protein was present in all samples and the gamma tubulin shows that protein was similar in all samples so as not to bias the results (Figure 4.18).

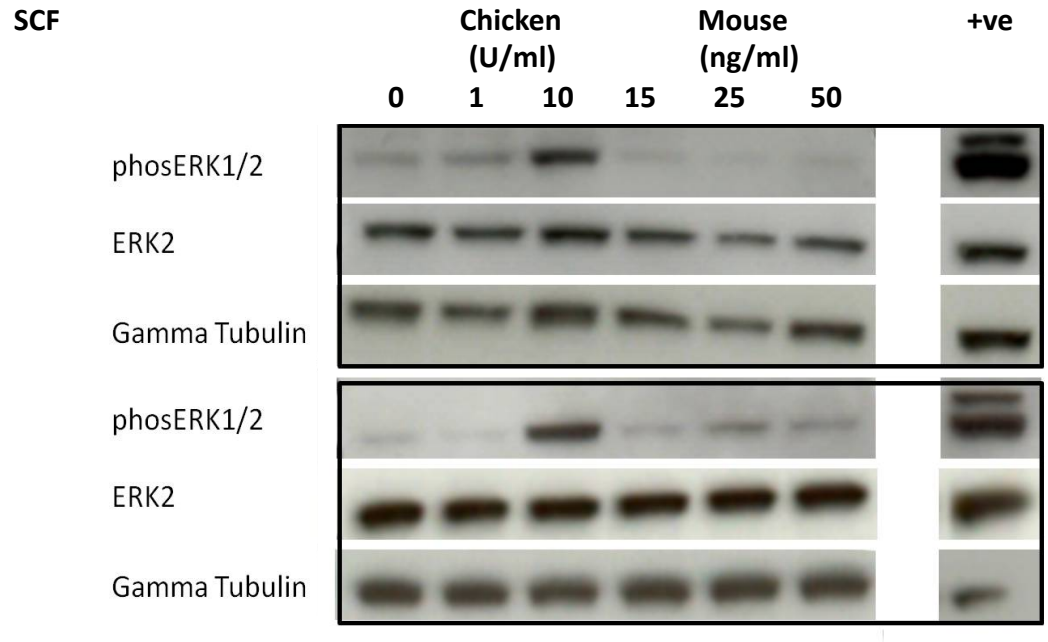


Figure 4.18 Analysis of ERK1/2 expression in PGCs cultured in the presence of SCF. Western analysis of total protein from chicken PGCs line 08.08.09, cultured in starvation medium for 4 hours and then in the presence of chicken SCF at (1 and 10 U/ml) and mouse SCF (15, 25 and 50ng/ml) for 15 minutes. The protein was probed for phosphorylated ERK1/2. ERK1/2 is phosphorylated under standard culture (Basic medium plus hFGF2) conditions (+ve). All samples were positive for total ERK2 protein (ERK2). Gamma tubulin shows that the protein was similar between samples. Experiments were carried out in replicated as represented by the individual panels

4.3.2.4 The effect of individual components of PGC culture medium on STAT3 phosphorylation

In section 4.3.1.5 it was observed that chicken PGCs grown in the presence of the JAK inhibitor, 420099 showed a significant reduction in proliferation compared to controls. This result indicated that by blocking JAK activity a down regulation of the JAK/STAT pathway was induced. In the following set of experiments, total cellular protein from chicken PGCs treated with individual components of the van de Lavoir medium, LIF and IGF1 were assessed for phosphorylation of STAT3. The proteins were probed with antibody phosphpoSTAT3 (Tyr705) (Cell signalling, Table 2.1) that detects STAT3 when phosphorylated at tyrosine residue 705 residue. As outlined in figure 4.2 the residue 705 is conserved between species. Unfortunately initial work to show that the phosphoSTAT3 antibody worked in chicken cells produced poor

results (Figure 4.2). It is shown in this chapter that the antibody does detect phosphoSTAT3 in chicken PGCs as evidenced by the control samples shown in all the STAT3 analysis. An antibody that detects unphosphorylated STAT3 (Table 2.1) was used to show the presence of total protein in each sample. STAT3 was unphosphorylated in all starved PGC control samples and phosphorylated under standard culture conditions (basic medium plus hFGF2).

4.3.2.4.1 Analysis of STAT3 phosphorylation in PGCs cultured in the presence of individual components of the culture medium

FGF2, FBS, chicken sera, SCF and BRL-conditioned medium are all components of standard culture medium. When the chicken PGCs were grown in basic medium plus hFGF2 STAT3 was phosphorylated (Figures 4.19-4.21) however it was not known which of the individual components induce this activation. Total protein extract from starved chicken PGCs, treated with either FGF2 (Figure 4.19a), SCF from chicken and mouse (Figure 4.19b), chicken serum (Figure 4.20a), FBS (Figure 4.20b) or BRL-conditioned medium (Figure 4.20c) were assessed by western blot analysis for the presence of phosphorylated STAT3. No phosphorylated STAT3 was detected in any of the four treatments. These findings indicated that none of these components of the medium were individually responsible for the STAT3 phosphorylation that was observed in the control cells.

4.3.2.4.2 Analysis of STAT3 phosphorylation in PGCs cultured in the presence of LIF or IGF1

Neither IGF1 nor LIF are added as purified factors to the medium in which chicken PGCs are cultured but it is likely that both factors are present in the sera or BRL-conditioned medium. LIF is known to activate the JAK/STAT pathway by binding the GP130/LIF-R heterodimer receptor molecule. This leads to phosphorylation of the STAT3 protein and signalling through the pathway. Analysis of the LIF amino acid sequences demonstrated that there is little homology between species, chicken, mouse, bovine or rat (Figure 4.15) and as discussed previously it is unlikely that there is functional conservation.

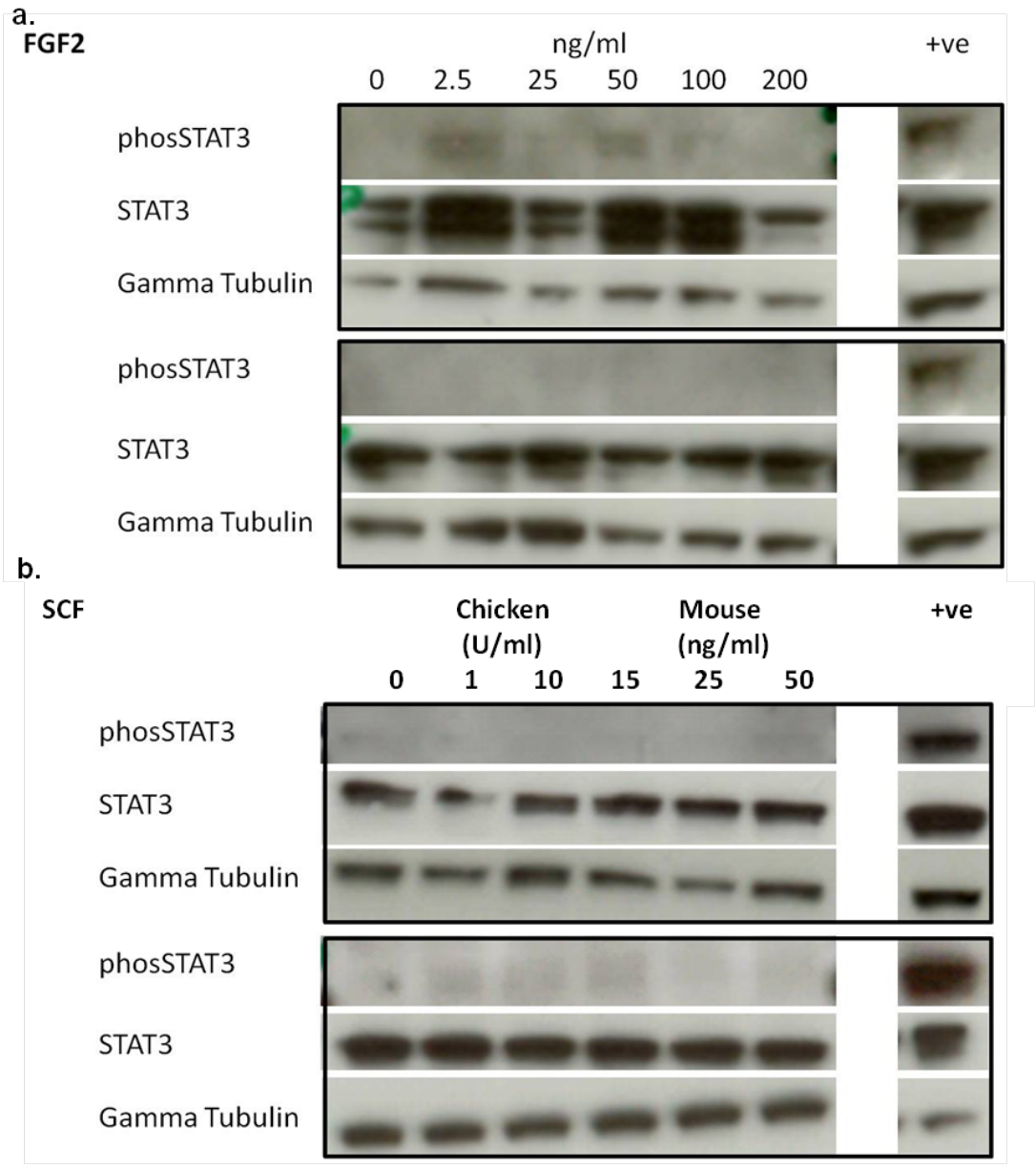


Figure 4.19 Analysis of STAT3 expression in PGCs cultured in the presence of hFGF2 or SCF. Western analysis of total protein from chicken PGCs line 08.08.09, cultured in starvation medium for 4 hours and then in the presence of (a) hFGF2 (2.5, 25, 50, 100 and 200ng/ml), (b) chicken SCF at (1 and 10 U/ml) and mouse SCF (15, 25 and 50ng/ml) for 15 minutes. The protein was probed for phosphorylated STAT3. STAT3 is phosphorylated under standard culture (Basic medium plus hFGF2) conditions (+ve). All samples were positive for total STAT3 protein (STAT3). Gamma tubulin shows that the protein was similar between samples. Experiments were carried out in replicated as represented by the individual panels.

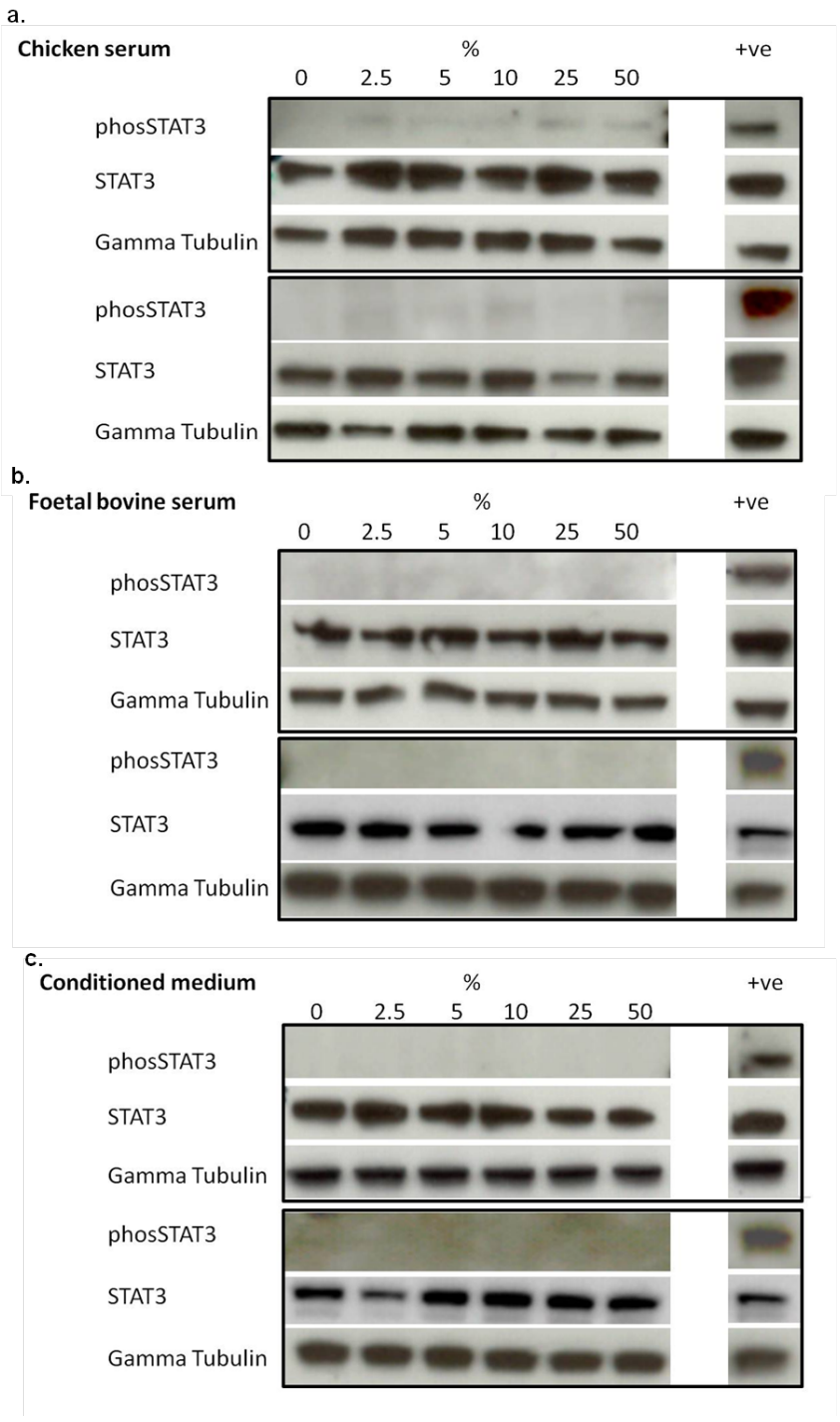


Figure 4.20 Analysis of STAT3 expression in PGCs cultured in the presence of sera or condtitioned medium. Western analysis of total protein from chicken PGCs line 08.08.09, cultured in starvation medium for 4 hours and then in the presence of of (a) Chicken serum (0, 2.5, 5, 10, 25, 50%), (b) FBS (0, 2.5, 5, 10, 25, 50%) or (c) BRL-conditioned medium (0, 2.5, 5, 10, 25, 50%)for 15 minutes. The protein was probed for phosphorylated STAT3. STAT3 is phosphorylated under standard culture (Basic medium plus hFGF2) conditions (+ve). All samples were positive for total STAT3 protein (STAT3). Gamma tubulin shows that the protein was similar between samples. Experiments were carried out in replicated as represented by the individual panels.

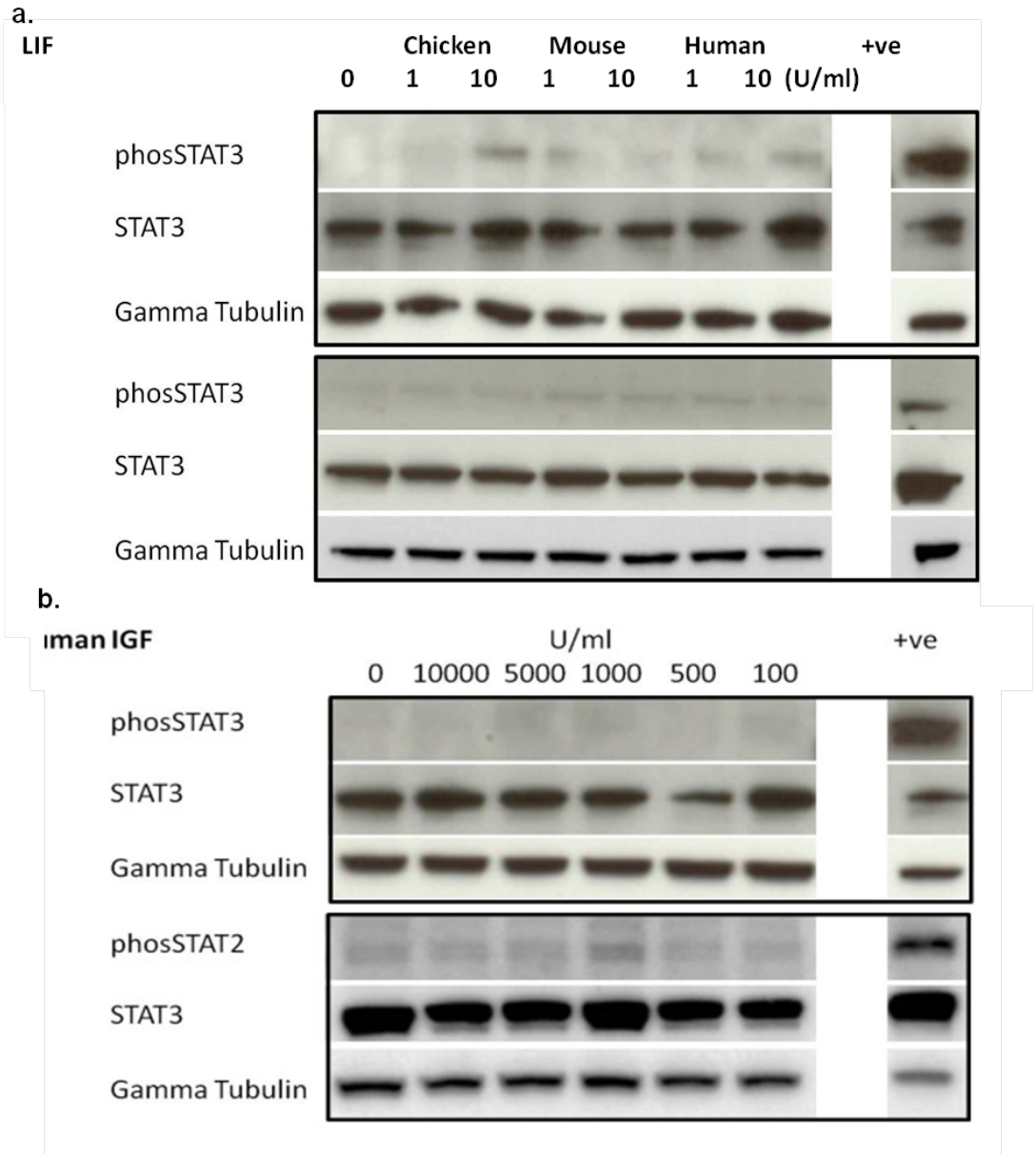


Figure 4.21 Analysis of STAT3 expression in PGCs when cultured in the presence of LIF. Western analysis of total protein from chicken PGCs line 08.08.09, cultured in starvation medium for 4 hours and then in the presence of (a) chicken, mouse and human LIF (1 and 10U/ml) or (b) hIGF1 (1-50U/ml) for 15 minutes. The protein was probed for phosphorylated STAT3. STAT3 is phosphorylated under standard culture (Basic medium plus hFGF2) conditions (+ve). All samples were positive for total STAT3 protein (STAT3). Gamma tubulin shows that the protein was similar between samples. Experiments were carried out in replicated as represented by the individual panels.

PhosphoSTAT3 was not detected in total cellular protein from starved chicken PGCs cells treated with either IGF1 or LIF. In the first LIF experiment there appears to be a low level of phosphorylated STAT3 detected in the samples from cells treated with cLIF at 10U/ml (Figure 4.21, top panel). However as it was not observed in the second experiment this may be the result of incomplete down regulation of the pathway prior to induction. Unphosphorylated STAT3 protein was shown to be present in all of the protein samples. It was unexpected that the cLIF did not induce the pathway and given that it was not observed to induce ERK1/2 phosphorylation in figure 4.17a this may indicate that the amounts of cLIF used were too low to induce pathway activation.

4.4 DISCUSSION

Van de Lavoir *et al* (2006) defined a culture system for propagation of chicken PGCs *in vitro* that was not easily recapitulated (Table 3.1). However as demonstrated in chapter 3 by focussing on the added growth factors it was demonstrated that addition of SCF to the culture medium was not a required for the isolation or propagation of chicken PGCs *in vitro*. In this chapter the van de Lavoir method has been scrutinised further to identify the status of signalling pathways, PI3K/AKT, MEK/ERK and JAK/STAT in chicken PGCs *in vitro* and the components of the medium that influence their activity. By gaining a better understanding of the requirements for chicken PGCs survival *in vitro* it is hoped that developments towards a serum and feeder free culture method can be achieved.

4.4.1 Signalling pathways required for PGC proliferation *in vitro*

When cultures of chicken PGCs were grown in medium containing small molecule inhibitors of PI3K (LY294002), MEK (PD0325901) and JAK (420099) it was demonstrated that cellular proliferation was significantly inhibited. The inhibitors were used to assess the function of signalling pathways PI3K/AKT, MEK/ERK and JAK/STAT.

4.4.1.1 Inhibition of the PI3K/AKT pathway

Using the small molecule inhibitor LY294002, signalling through PI3K was blocked resulting in a significant ($P<0.01$) inhibition in chicken PGC proliferation compared to the control wells (Figure 4.3). This result showed that PI3K mediated signalling was essential for chicken PGC proliferation in culture. This result differs from what has been observed in the mouse. De Miguel *et al*. (2002) demonstrated that addition of the PI3K inhibitor LY294002 to mouse PGCs cultures had no effect on germ cell number (De Miguel *et al*. 2002). These differences could be attributed to the difference in proliferation rates, two-fold increase in the mouse PGC cultures in comparison to the twenty-fold increase reported here in the chicken PGCs over the course of seven days.

4.4.1.2 Inhibition of the MEK/ERK pathway

It has been shown here that MEK inhibitor PD032590 effectively down regulates ERK1/2 phosphorylation (Figure 4.4c) and when cultured chicken PGCs are treated with the inhibitor the cells do not proliferate at the same rate as controls (Figure 4.4b). Inhibition of MEK resulted in a more than eight-fold decrease in cellular proliferation rate in comparison to cells grown in basic medium plus hFGF2. This result is the same as was observed in mouse PGCs treated with MEK inhibitors. When mouse PGCs are cultured *in vitro* in the presence of or exposed *in vivo* to MEK inhibitors a significant ($P<0.05$ and $p<0.01$) reduction in total PGC number compared to controls is observed (De Miguel *et al*. 2002; Takeuchi *et al*. 2005). The findings presented here indicate that the MEK/ERK pathway is essential for chicken PGC propagation *in vitro*. FGF2 and SCF, both added components of the van de Lavoir culture method, have a role in activating the MEK/ERK pathway. These results support the recommendation for the addition of the growth factors to the culture medium although it was demonstrated in chapter 3 that addition of FGF2 alone is sufficient.

4.4.1.3 Inhibition of the JAK/STAT pathway

The JAK\STAT pathway, involved in the maintenance of ES cell pluripotency was shown here to be essential for maintenance of chicken PGCs in culture. In an assay that was initiated with 1000 chicken PGCs per well it was observed that after seven days in culture there was significantly ($P<0.01$) more cells in the control wells compared to inhibitor treated wells (Figure 4.5). This indicated that the JAK/STAT signalling pathway is essential for propagation of chicken PGCs *in vitro*. The results presented here are similar to those observed in mouse PGC culture in the presence of LIF. When LIF is present in mouse PGC culture medium, meiosis and apoptosis is inhibited (Chuma and Nakatsuji 2001; Farini *et al*. 2005). Although this result is induced by LIF, LIF activates the STAT pathway. It is therefore likely that when the JAK/STAT pathway was inhibited in the chicken PGCs the difference in cell number resulted from increased apoptosis in the inhibitor treated cells.

The data produced from the inhibition assays is from examination of signalling pathways in isolation but pathway interaction could influence any results. This includes activation of the ERK1/2 pathway through PI3K by SCF/c-kit interaction, which has been demonstrated in haematopoietic progenitor cells (Wandzioch *et al*. 2004). Signalling via the FGF receptors has also been demonstrated to actively induce PI3K signalling in cell lines and during early vertebrate development (Ong *et al*. 2001; Böttcher and Niehrs 2005). To gain a better understanding of what extracellular factors activate these signalling pathways in the chicken PGCS a more detailed examination of the pathways was required.

4.3.2 The effect of culture medium components on pathway activation in chicken PGCs *in vitro*

The experiments described here using small molecule inhibitors of PI3K, MEK and JAK clearly showed that the three signalling pathways, PI3K, ERK1/2 and JAK/STAT are essential for maintenance of chicken PGCs in culture. To define how these pathways are stimulated in chicken PGCs in culture, the van de Lavoir culture medium was dissected and the individual components investigated. Using western blot analysis components of the culture medium and several growth factors were examined for their ability to induce activation of the individual pathways in cultures of chicken PGCs grown in starvation medium for four hours.

4.3.2.1 FGF2 induced activation of signalling pathways

As was shown (Figure 4.13) the FGF2 amino acid sequence is conserved between chicken and humans and may be conserved at a functional level. This prediction was supported by the results seen in chapter 3 where it was shown that addition of hFGF2 to cultures improved chicken PGC isolation from embryonic blood. Choi *et al* (2010) also showed that chicken PGC proliferation was increased when hFGF was added to cultures. In the induction experiments it was shown that when starved chicken PGCs were cultured in the presence of hFGF2 only the MEK/ERK pathway was activated as evidenced by the detection of phosphorylated ERK in the protein extracted from the cell. This result is unsurprising as FGF2 is well documented as an inducer of both

the MEK/ERK pathway (Katz *et al* 2007). Interestingly FGF2 has also been shown to activate the PI3K/AKT pathway in mouse EG cells (Kimura *et al*. 2007). The lack of FGF2 induced PI3K/AKT activation may be due to a control mechanism active within the PGCs inhibiting chicken PGC dedifferentiation.

4.3.2.2 SCF induced activation of signalling pathways

SCF induced signalling promotes proliferation and survival in the culture of mouse PGCs (Dolci *et al*. 1991; Godin *et al*. 1991; Matsui *et al*. 1991; Manova *et al*. 1992; Pesce *et al*. 1993). However is was shown by Choi *et al* (2010) and in chapter 3 that chicken PGCs can be propagated in culture without the addition of SCF to the medium. SCF\c-Kit interaction can induce both the PI3K/AKT (Sette *et al*. 2000; De Felici 2000; Liu *et al*. 2007) and MEK/ERK (Dolci *et al*. 2001) pathways. It was predicted that given the low level of sequence homology between chicken and mouse SCF (Figure 4.7) it would be unlikely that the SCFs would be functionally conserved. This was validated by the western blot analysis of protein extracted from chicken PGCs cultured in the presence of mouse or chicken SCF. The results showed that only the SCF from chicken induced activation of the PI3K/AKT (Figure 4.6a) and the MEK/ERK (Figure 4.18) pathway in the chicken cells. The lack of phosphorylated AKT or ERK1/2 in the mSCF induced cells is likely to be due to a lack of functionality of the mouse growth factor in the chicken cell line. In the published culture protocol the source of SCF is omitted, as it was added in quantified amounts it was assumed that the source must have been commercially available and therefore not chicken. Although it has been documented that removal of additional SCF and FGF2 from the culture medium results in the dedifferentiation of the PGCs to EG cells (van de Lavoir *et al*. 2006) it is possible that removal of SCF alone would not have affected the cultures, which is supported by the data presented in chapter 3.

4.3.2.3 IGF1 induced activation of signalling pathways

IGF1 induced signalling pathway is essential for growth and development in vertebrates and is evolutionarily conserved (LeRoith *et al* 2000; Nakae *et al.* 2001). IGF1 signalling can activate the PI3K/AKT and MEK/ERK pathways (Dupont and

LeRoith 2001; Schlueter *et al*. 2007a; Schlueter *et al*. 2007b). IGF1 induced signalling is involved PGC migration, specification and proliferation (Schlueter, Sang, *et al*. 2007; Schlueter, Peng, *et al*. 2007). In the van de Lavoir culture method IGF1 is not added as a purified factor. However it has been shown here that when the protein from starved cells cultured in the presence of hIGF1 was analysed, phosphorylated AKT (Figure 4.6b) but not phosphorylated ERK1/2 (Figure 4.17b) was detected. The lack of functional conservation of SCF, the phosphorylation of AKT detected when analysing other components of the culture medium that do not contain chicken SCF suggest that IGF1 in these components may be the factor activating the PI3K/AKT pathway in chicken PGCs *in vitro.*

4.3.2.4 LIF induced activation of signalling pathways

LIF is an important cytokine that promotes survival and self-renewal of PGCs (reviewed by Katz *et al*. 2007). The MEK/ERK and JAK/STAT pathway are both induced by LIF. Although not added to chicken PGC culture medium as a purified factor LIF is secreted by BRLs into the conditioned medium (Chuma and Nakatsuji 2001). Addition of LIF to culture medium has been implicated in the differentiation of PGCs to EG cell in mouse, rabbit and chicken (Matsui *et al*. 1992; Park and Han 2000; Kakegawa *et al*.2008) However the presence of a LIF-expressing feeder layer has been shown to have inhibitory effects on mouse PGC differentiation too (Chuma and Nakatsuji 2001; Farini *et al*. 2005). When LIF from chicken mouse and human were tested individually no phosphorylation of either ERK1/2 or STAT3 was detected, for the mouse LIF this is expected as it has been documented that mouse LIF is not functional in chicken cell lines (Horiuchi *et al*. 2004). As human LIF shares greater similarity to mouse than chicken it is likely that the hLIF is also not functionally conserved with cLIF and so did not induce pathway activation in the chicken PGCs. Although this data highlighted the lack of functional conservation between species it does not account for the absence of phosphorylated ERK1/2 and STAT2 in the samples treated with chicken LIF. The lack of induction in either pathway suggests that the chicken LIF used may have been at concentrations too low to be effective. The chicken LIF used, was stored at 4^{o}C for several months, this may

have resulted in a reduction in the cytokine activity. Chicken LIF is currently not commercially available and as yet there is no antibody to carry out ELISA for the quantification of chicken LIF, which is produced from transfected rat fibroblast cells. Without proper validation of chicken LIF it is not possible to draw conclusions from the absence of pathway activation.

4.3.2.5 Chicken sera and FBS induced activation of signalling pathways

Analysis of protein from starved chicken PGCs cultured in the presence of chicken sera or FBS only detected phosphorylated ERK1/2 (Figure 4.16). Both chicken sera and FBS contain unknown levels of various growth factors, one or more of which must be responsible for inducing the MEK/ERK pathway. Likely factors include FGFs, SCF and IGF1. As chicken sera induced neither the PI3K/AKT nor the JAK/STAT pathway it is unlikely that cSCF or cIGF1, known activators of these pathways, are inducing phosphorylation of ERK1/2. This indicates that FGF2 may be inducing the activation. This is supported by an identical induction resulting from using FBS induce pathway activation. Unlike FGF2, SCF does not appear to be functionally conserved between species. Therefore it is more likely that FGF2 present in the FBS is inducing ERK1/2 phosphorylation because if it was IGF1 activation of the PI3K/AKT pathway, which is induce by hIGF1, would be expected. Although FGF2 has been shown to activate PI3K/AKT signalling (Kimura *et al* 2007) it was shown here that purified hFGF2 did not induce AKT phosphorylation in the starved chicken PGCs. Taking this into consideration it further supports FGF2 as the component of chicken sera and FBS causing the observed activation of the MEK/ERK pathway.

4.3.2.6 BRL-conditioned medium induced activation of signalling pathways

BRL-conditioned medium has been shown to contain several growth factors, LIF, IGF1 and SCF (Smith *et al*. 1988; Zsebo *et al*. 1990). BRL-conditioned medium was shown to induce AKT phosphorylation (Figure 4.9) and ERK1/2 phosphorylation (Figure 4.14b). As SCF and LIF are not conserved between chicken and mouse it is

unlikely that SCF and LIF secreted from the BRL cells into the conditioned medium are inducing phosphorylation of either protein. This suggests that induction of the PI3K/AKT pathway by conditioned medium is due to the IGF1 and not the SCF that is excreted by the BRL cells. However IFG1 when added as a purified factor did not induce the MEK/ERK pathway (Figure 4.17b), this indicates that the phosphorylation of ERK1/2 is mediated by another factor present in the BRL-conditioned medium. From the data presented here it should be considered that the ERK1/2 phosphorylation is mediated by FGF2 provided by the FBS that is a component of BRL-conditioned medium.

4.3.2.7 Individual components of PGC culture medium do not stimulate STAT3 phosphorylation

The inhibition of PGC propagation *in vitro* observed in the chicken PGC cultures cultured in the presence of the JAK inhibitor and the detection of phosphorylated STAT3 control cells showed that the JAK/STAT pathway was active and essential for PGC propagation in culture. However JAK/STAT pathway activation evidenced by the detection of phosphorylated STAT3 was not observed in any of the protein samples tested. These results indicate that no single component of the culture medium is responsible for activation of the pathway in the cultured chicken PGCs. A distinct lack of induction from any individual component, the obvious requirement for JAK/STAT signalling represented in the inhibitor experiments and the presence of phosphorylated STAT3 under normal conditions is interesting.

4.5 CONCLUSIONS

In conclusion the PI3K/AKT pathway plays a vital role in the proliferation of chicken PGCs *in vitro* but is perhaps stimulated by IGF1 and not SCF. SCF therefore may not be essential for the *in vitro* propagation of chicken PGCs. MEK/ERK1 signalling has also been demonstrated to be crucial for cultured chicken PGC survival and is likely to be mediated by FGF2 and FGF receptor interaction, although perhaps not exclusively by this trigger. Although JAK/STAT signalling is activated in chicken PGCs cultured in basic culture medium plus hFGF and identified to be required for the active proliferation of chicken PGCs in culture, no individual component of the culture medium stimulated its activation. Although STAT3 is phosphorylated in the cells, LIF activation of JAK can also stimulate the MEK/ERK pathway (Burdon *et al*. 1999). Perhaps then inhibition of JAK may be resulting in a down regulation of the MEK/ERK pathway instead of JAK/STAT. This would have the same observed result on cell propagation as validated by the results from the MEK inhibitor experiment. This could be confirmed by western blot analysis of chicken PGCs to detect down regulation of either the MEK/ERK of JAK/STAT pathways in response to JAK inhibitor treatment. JAK/STAT signalling may therefore not be essential and this may explain why it is not stimulated by individual components of the medium. Alternatively the chicken PGCs themselves may produce high levels of chicken LIF under normal culture conditions that result in the phosphorylation of STAT3. Without a suitable ELISA for chicken LIF it is as yet impossible to elucidated whether or not the chicken PGCs are expressing LIF at levels capable of inducing JAK/STAT signalling.

CHAPTER 5: GENETIC MANIPULATION OF CULTURED CHICKEN PRIMORDIAL GERM CELLS

5.1 INTRODUCTION

There is a wide range of applications for transgenic chickens in academic research, industry and agriculture but the methods by which they are produced have been difficult to develop and challenging to execute. As discussed in the general introduction (1.4) the techniques for the production of transgenic mammals could not be adapted for chickens due to the differences in the reproductive system and the requirement for the embryonic development to occur within a shelled egg. Methods that have been used successfully to produce transgenic birds are injection of a gene of interest into the zygote which requires sacrificing a laying hen each time and viral vector mediated gene transfer by injection into the subgerminal cavity of the stage X (EG&K) embryo. However low efficiency and a time consuming process from start to production of a transgenic keeps researchers striving for a simpler cell based method. Cell based methods offer the ability to transfect and select for specific integrations or carryout gene targeting. The successful derivation of chicken ES cells held great promise but unlike in mammals only somatic chicken chimeras could be produced. The method for the prolonged culture of chicken PGCs *in vitro* that has been established may provide the cell-based method for manipulation of the chicken genome that is desired (van de Lavoir *et al*. 2006).

5.1.1 Manipulation of PGCs

Previous attempts to manipulated chicken PGCs for the purposes of transgenic bird production have proved relatively unsuccessful. As discussed in the general introduction (1.4.4.1) retroviral-vectors have been used to manipulate freshly isolated chicken PGCs and cells maintained in culture for up to two and a half days (Vick *et al*. 1993; Thoraval *et al*. 1995; Motono *et al*. 2010). Although transmission frequency of the transgene was shown to be up to 7% from some chimeras, retroviral vectors will only integrate into dividing cells and are limited in the amount of DNA

they can deliver. There is currently no published work describing retroviral vector transduction of actively dividing chicken PGCs in culture.

5.1.1.1 Modification of PGCs using plasmid DNA

As outlined in the general introduction (1.5.4.1) van de Lavoir *et al*. (2006) tried genetically modifying chicken PGCs *in vitro* by electroporation of DNA plasmids carrying several different reporter cassettes, including a GFP (green fluorescent protein) expressing transgene, β-actin-neo and ERNI-puro. However initial attempts to produce transgenic cells from any of these vectors were unsuccessful. This was attributed to the gene silencing as a result of epigenetic modification. Insulator sequences (HS4) act as a barrier to gene silencing (Burgess-Beuss *et al*. 2002). After insertion of these sequences into the plasmids to flank the reporter cassettes transgenesis of the PGCs was achieved. Van de Lavoir *et al* (2006) report that they successfully stably transfected PGCs with four different constructs into which the insulators sequenced had been inserted. PGCs expressing the GFP reporter gene were shown to still form functional gametes.

Although stable transfection was achieved the efficiency was low. Leighton *et al* (2006) reported that in PGCs transfected with a CAG-neo construct flanked with the insulator sequences that only one positive clone was identified. This was the result from a large number of electroporation experiments resulting in a stable transfection rate of $5x10^{-7}$%. Despite the low stable transfection frequencies the modified cells were able to transmit through the germline at frequencies from less than 1% to as much as 96%.

5.1.1.2 Modification of PGCs using the ΦC31 integrase system

As outlined in the general introduction (1.5.4.2) Leighton *et al*. (2006) utilised the ΦC31 integrase system to improve the frequency with which cultured chicken PGCs could be modified. The ΦC31 integrase system works by mediating integration of attB-containing plasmids into pseudo attP sites in the chicken genome. The ΦC31 integrase system was tested using several different reporter constructs with and

without insulator sequences inserted into an attB-containing plasmid. Cells were electroporated with the plasmid carrying the reporter construct and a plasmid designed to express ΦC31 integrase. A maximum stable transfection rate of 0.001% was achieved in the presence of the insulator sequences and a stable transfection frequency of 0.0004% in the constructs where the HS4 was removed. This higher rate of stable transfection resulting from the presence of insulator sequences supported the argument for gene silencing having an adverse effect on the manipulation of culture chicken PGCs. However even without insulators the stable transfection efficiency using the ΦC31 integrase system was a 100-fold higher than using DNA plasmid plus insulator constructs. This highlights the efficiency of integrase mediated genetic manipulation. From these results it indicated that efficiency of chicken PGC genetic manipulation could in part be improved by using a more efficient gene delivery system.

5.1.2 Selection of transposon derived vectors

Transposon-derived vectors have been used efficiently in the transgenesis of many vertebrate embryos and cells lines. Transposons and transposon-derived vectors have been described in the general introduction (1.6). The vectors work via a binary system of a "donor" plasmid carrying a gene of interest and a second "helper" plasmid carrying the transposase gene (Figure 1.15). Transposition is controlled by expression of the transposase in trans. For the following experiments two different transposon-derived vectors, one from piggyBac and one from Tol2, were chosen. Both vector systems have been shown to work efficiently in mammalian cell lines and in the chicken. The *Tol2* vector used here was demonstrated by Wang *et al*. (2007) to efficiently transfect foreign DNA into the chicken cell line DF1 and into cells of the chicken PSM. The piggyBac vector has also been used to stably transfect the developing spinal cord in the chicken embryo and analysis of transfected DF1s confirmed that stable expression was due to transposase mediated integration of the piggyBac confirmed by the presence of TTAA repeats at either end of the site of integration. This is indicative of piggyBac transposon integration. These experiments

highlight the potential for each vector as an efficient method for the genetic manipulation of the chicken PGC genome.

The aim of the experiments described in this chapter was to find a more efficient method for the transgenesis of cultured chicken PGCs. This required evaluation of vectors derived from the DNA transposons, *Tol2* and piggyBac ability to produce stably transfected chicken PGCs *in vitro*. This involved cloning of transposon-vectors carrying a reporter construct, testing it in a chicken fibroblast cell line and then assessing the efficiency of transgenesis in chicken PGCs. The effect on transposon-mediated genetic modification on germline transmission was also assessed.

5.2 AIMS

1. To demonstrate that transposition of piggyBac and *Tol2* vectors in chicken DF-1 fibroblasts.
2. To measure the efficiency and stable transfection of chicken PGCs, using the *Tol2* and piggyBac transposon vectors.
3. To demonstrate that PGCs genetically modified using piggyBac retain the ability to migrate to the developing gonad
4. To demonstrate that PGCs genetically modified using piggyBac retain the ability to form functional sperm.

5.3 RESULTS

5.3.1 Generation of transposon-derived vectors containing identical reporter cassettes

Two transposon-derived vectors were tested. PiggyBac (PB transposon; Appendix 1) provided by Dr Wei Wang and Dr Pentao Liu (Wang *et al*. 2008) and *Tol2* (pT2K-CAGG-GFP; Appendix 1) from Dr Koichi Kawakami and Dr Yoshiko Takahashi (Sato *et al*. 2006). The inverted terminal repeats (ITR) of *Tol2* and PiggyBac in the transposon vectors were 550bp and 236bp, respectively.

5.3.1.1 Molecular cloning of piggyBac vector

The pCAGG-GFP-IRES-PURO (pCGIP, Appendix 1) vector was digested with restriction enzymes (Materials and methods 2.6.9) Bam HI and Sal I to isolate the 4.3kb CAGG-GFP-IRES-PURO (CGIP) reporter cassette. The reporter cassette consisted of a hybrid promoter CAGG, containing the CMV-IE enhancer fused to the chicken β-actin promoter and the first intron. The CAG promoter was used to drive expression of fluorescent reporter eGFP fused to IRES (internal ribosomal entry site) and puromycin selectable marker. The isolated DNA was then inserted into the piggyBac transposon vector (PB Transposon) that had been linearised by digestion with Bam HI and Sal I. The resulting 8.2kb vector is referred to throughout this chapter as PB-CGIP, (Figure 5.1a). Previously reported attempts to transfect exogenous DNA plasmids carrying resulted in genes in to cultured chicken PGCs reported silencing of the transgene after integration. This problem was overcome by the insertion of insulating elements around the gene of interest (van de Lavoir *et al*. 2006). The insulator sequence (HS4, Appendix 1) used was isolated and provided by Dr Adam West (Recillas-Targa *et al*. 2002). To counteract any effects of gene silencing may have on the transposon mediated insertion, HS4 sequences were inserted into the piggyBac vector, PB-CGIP. The HS4 elements were inserted into the PB-CGIP vector flanking the receptor cassette. The element was prepared by cutting with Kpn I and inserted into the vector, first at the 3' end of the reporter cassette by cutting PB-CGIP with Pst I (Appendix 1). HS4 was then inserted at the 5'

end using Spe I to linearise the PB-CGIP vector (Appendix 1) that had the 3' HS4 element inserted. The resulting vector is referred to as PB-CGIP-HS4 (Figure 5.1c).

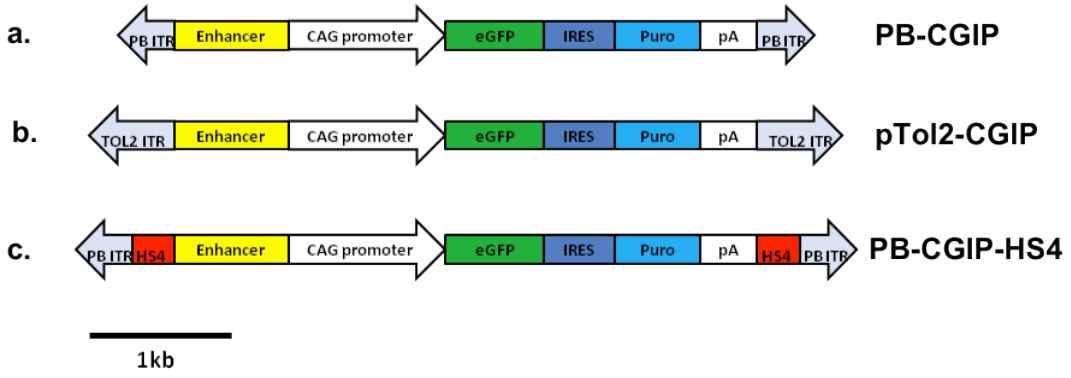


Figure 5.1 Transposon-derived vector construction. (a) Illustrations of the vectors constructed, PB-CGIP, (b) pTOL2-CGIP and (c) PB-CGIP-HS4. Inverted terminal repeats (Grey), internal ribosomal entry site (dark blue), puromycin selectable marker (light blue), poly A tail (white) and insulator elements (Red).

5.3.1.2 Molecular cloning of *Tol2* vector

The *Tol2* vector (pT2K-CAGG GFP) already consisted of a CAGG driven GFP so it was only the IRES-PURO selection of the reporter cassette that needed to be inserted. The IRES-PURO was isolated from pCGIP by digesting with the Xho I restriction enzyme (Appendix 1) and the isolated sequence inserted into pT2K-CAGG GFP that had been digested using Xho I as well (Appendix 1) (Materials and methods 2.6.9 and 2.6.11). The resulting vector (Figure 5.1b) consisted of *Tol2* inverted repeats flanking a cassette identical to the one inserted into PB-CGIP. The vector is referred to as pTol2-CGIP throughout the rest of this chapter.

Insertion of the reporter cassette between the ITRs of the transposon vectors allowed for stable genomic transfection to be measured. This was done by the observation of GFP expression and the puromycin selectable marked was used to kill cells into which the reporter construct had not been stably transfected.

5.3.2 Comparison of piggyBac and tol2 vector mediated transfection in chicken DF-1 cells

Having inserted identical reporter cassettes into the transposon-derived vectors it was possible to make direct comparisons between the transfections efficiencies for each vector. The three transposon-derived vectors (Figure 5.1) were initially tested in the chicken fibroblast cell line, DF1. These experiments were carried out to establish that the vectors created were functional in a chicken cell line. Transfections were carried out using FuGENE transfection reagent on six well plates (Materials and methods 2.7.5). Each well was treated with equal quantities of DNA; 2µg of pTol2-CGIP or PB-CGIP and 2µg of transposase or control plasmid.

5.3.2.1 Transient transfection efficiencies

Cells were assayed visually and by FACS analysis 72 hrs post transfection to estimate the proportion of GFP expressing cells. At 72hrs post transfection no visual difference could be observed between cells transfected with or without transposase for all three vectors (Figure 5.2). When cells were transfected with PB-CGIP minus the transposase plasmid, 24.3% of cells were GFP-expressing, this was higher than the 13.3% of cells that were transfected using PB-CGIP plus transposase plasmid. 24.1% of cells were transiently transfected by PB-CGIP-HS4 minus transposase plasmid compared to 7.9% when the transposase plasmid was co-transfected. The transient transfection rates for the pTol2-CGIP vector plus and minus transposase plasmid were 19.8% and 23.2% respectively. Standard posthoc pairwise Tukey comparison revealed that transient transfection rates were significantly lower in the presence of transposase ($p=0.005$). This effect on transfection efficiency did not differ between transposon vectors ($p=0.086$).

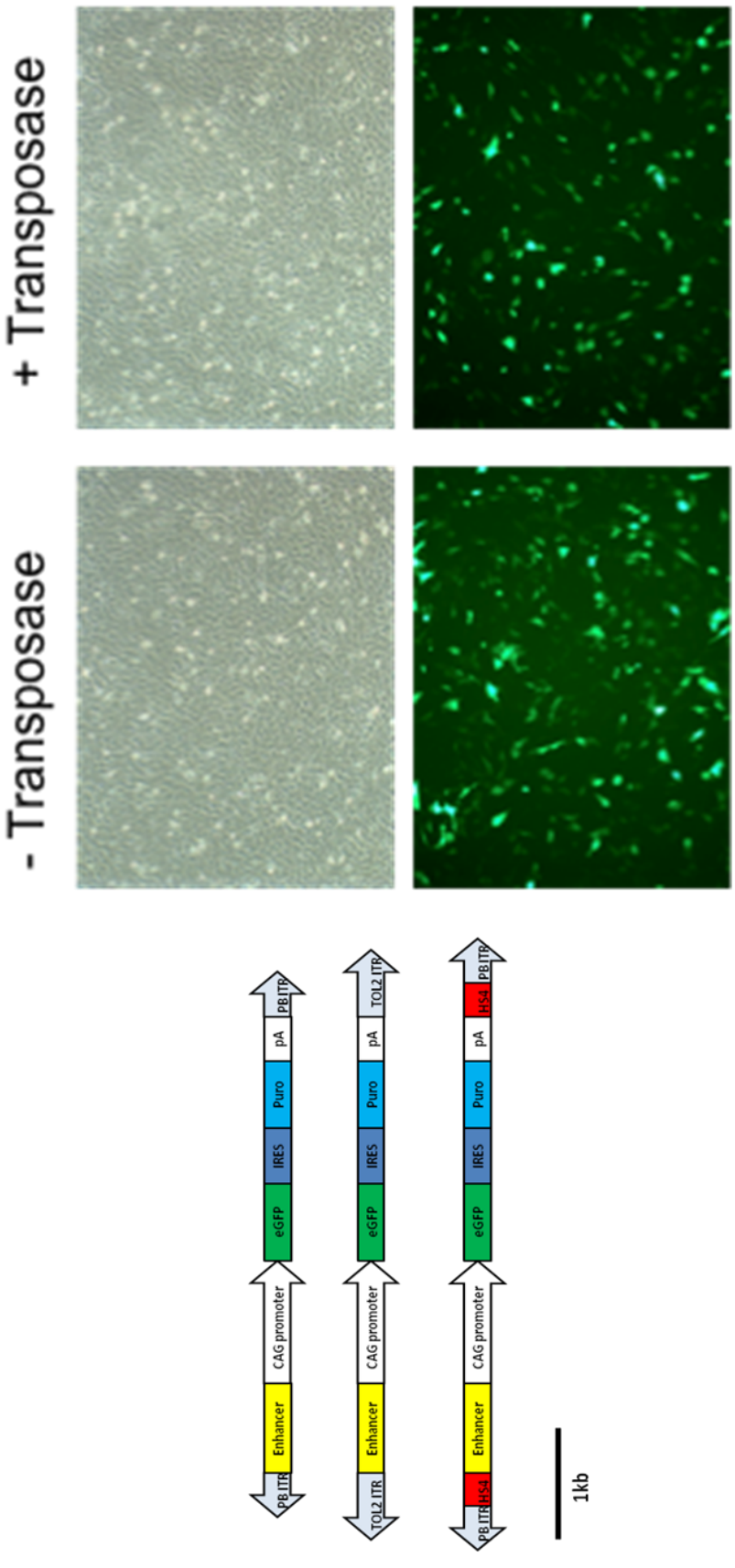


Figure 5.2 DF1 cells expressing GFP 72 hours post transfection. (a) Representative images of DF-1 cells expressing GFP 72 hrs after transfection with any one of the transposon vectors plus or minus transposase.

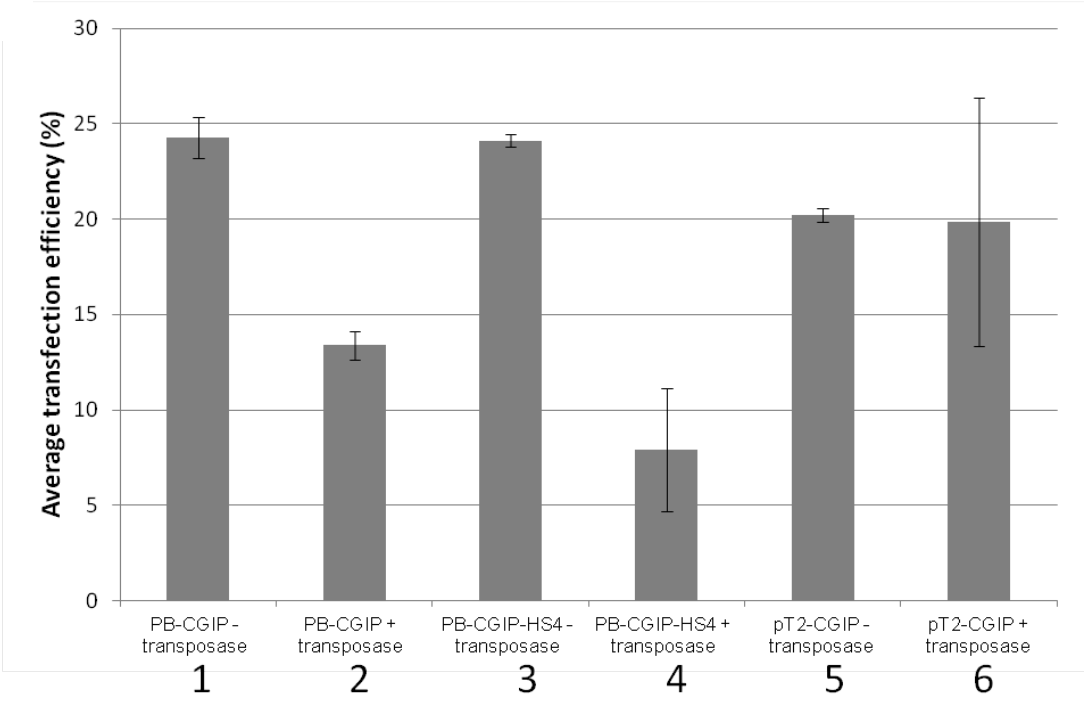


Figure 5.3 Transient transfection efficiencies in chicken DF-1 cells. Graph showing the average transfection efficiencies of the six different transfections assessed: **1** PB-CGIP minus transposase; **2.** PB-CGIP plus transposase; **3.** PB-CGIP-HS4 minus transposase; **4.** PB-CGIP-HS4 plus transposase; **5.** pTol2-CGIP minus transposase and **6.** pTol2-CGIP plus transposase. Error bars, SEM. ***, P <0.001

5.3.2.2 Stable transfection efficiencies

At three weeks the cells were FACS analysed again to estimate the proportion of GFP expressing cells. Stable transfection rates were corrected for transfection efficiency (Materials and methods 2.8.1) and the data represents at least three independent experiments per vector. Figure 5.4 shows a graph of the average stable transfection efficiencies calculated for each group of transfections. Only 0.8% of cells transfected with PB-CGIP minus the transposase plasmid were GFP-expressing, this was lower than 4.6% of cells that were stably transfected using PB-CGIP plus transposase plasmid. 10.1% of cells were stably transfected using PB-CGIP-HS4 plus transposase plasmid compared to 1.3% in the absence of transposase plasmid. The stable transfection rates for the pTol2-CGIP vector plus and minus transposase plasmid were different at 34.1% and 1.0% respectively.

Overall an increase in the stable transfection frequency in the presence of transposase for all three transposon vectors was observed ($p<0.001$). There was also a statistically significant difference between this increase and the stable transfection efficiency of each transposon vector. Considering only the transposon vectors in the presence of transposase data, standard posthoc Tukey pairwise comparison revealed a significant difference between all three transposon vectors ($p<0.001$). These results demonstrate that both piggyBac- and *Tol2*-derive vectors can be used efficiently to integrate genes into chicken cells. As a significant increase in stable integration rate was observed between PB-CGIP and PB-CGIP-HS4 ($p<2e-16$) this result suggests that in DF1 that transgene silencing may be having an effect on the PB-CGIP vectors transposase mediated stable integration rates.

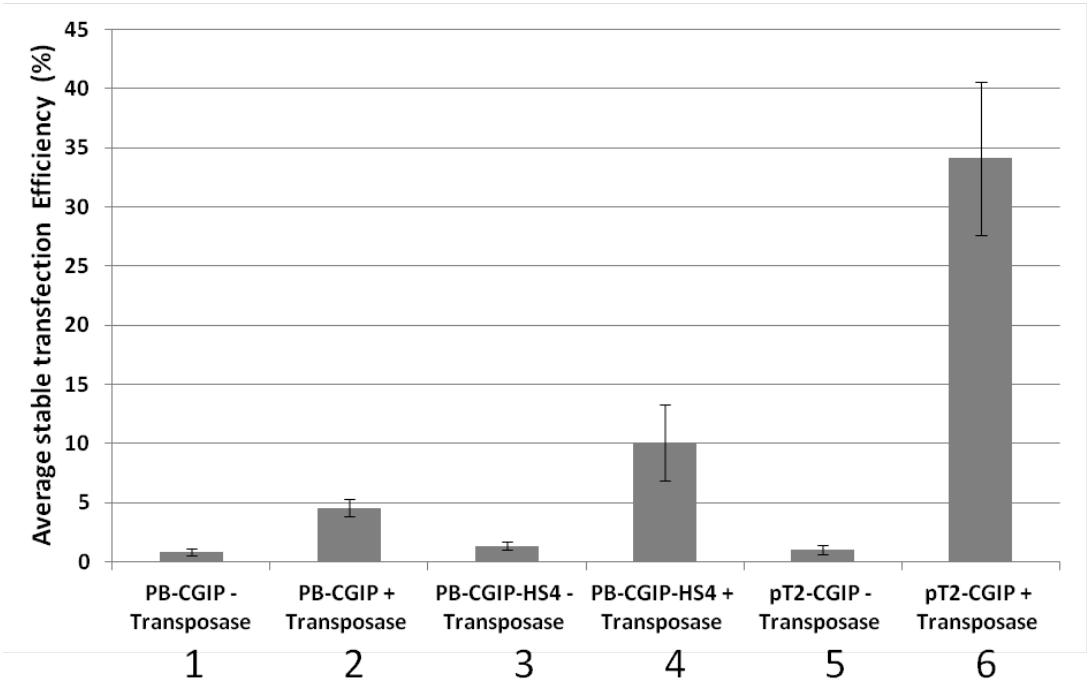


Figure 5.4 Stable transfection efficiencies in chicken DF-1 cells. Graph showing the average stable transfection efficiencies of the six different transfections assessed: **1** PB-CGIP minus transposase; **2.** PB-CGIP plus transposase; **3.** PB-CGIP-HS4 minus transposase; **4.** PB-CGIP-HS4 plus transposase; **5.** pTol2-CGIP minus transposase and **6.** pTol2-CGIP plus transposase. Error bars, SEM. ***, $P < 0.001$

5.3.3 Comparison of piggyBac and *Tol2* vector mediated transfection in chicken primordial germ cells

The results from the DF-1 cell transfection experiments indicated that both *Tol2* and piggyBac-derived vectors were functional in chicken cell lines. To determine if these transposons could be used to stable transfect chicken PGCs the three vectors were assayed for stable transfection efficiency in PGCs. Two PGC lines, 08.08.09 and 193-3 (cultured by Dr M McGrew) were transfected with PB-CGIP, PB-CGIP-HS4 or pTOL2-CGIP in the presence or absence of transposase plasmid. Transfections were carried out using the DMRIE- C transfection reagent, following the protocol optimized for use on non-adherent cells (Materials and methods 2.7.6). The experiments were carried out on samples of approximately 30,000 PGCs not enough to run FACS analysis due to difficulties in increasing cell numbers to amounts that could be used for FACS and the expense of doing so. No GFP expression was observed three weeks post-transfection in cells transfected with the transposon-derived vectors in the absence of transposase plasmid (Figure 5.5a) whilst in the presence of transposase plasmid stable expression of the reporter gene for both the piggyBac and *Tol2* transposons was achieved (Figure 3b and c). The transfected cells were counted by eye at three days and three weeks post-transfection to estimate the proportion of GFP-expressing cells. Stable transfection rates were calculated based on the total number of cells initially transfected and the proportion of cells still expressing GFP after three weeks (Materials and methods 2.8.1). Figure 5.5d shows that in the absence of transposase no PGCs are stably transfected with the reporter construct regardless of transposon vector. The graph also shows that 12.5% of cells transfected with PB-CGIP plus transposase plasmid stably expressed GFP, this was more than the 4% of GFP-expressing cells observed in the PB-CGIP-HS4 plus transposase plasmid cultures. 35% of cells transfected with the pTOL2-CGIP vector plus transposase plasmid still expressed GFP three weeks post transfection.

Statistical analysis of this data reveals that PGC stable transfection frequencies increased in the presence of transposase for all three transposon vectors ($p<0.001$). However there was also a statistically significant difference between this increase

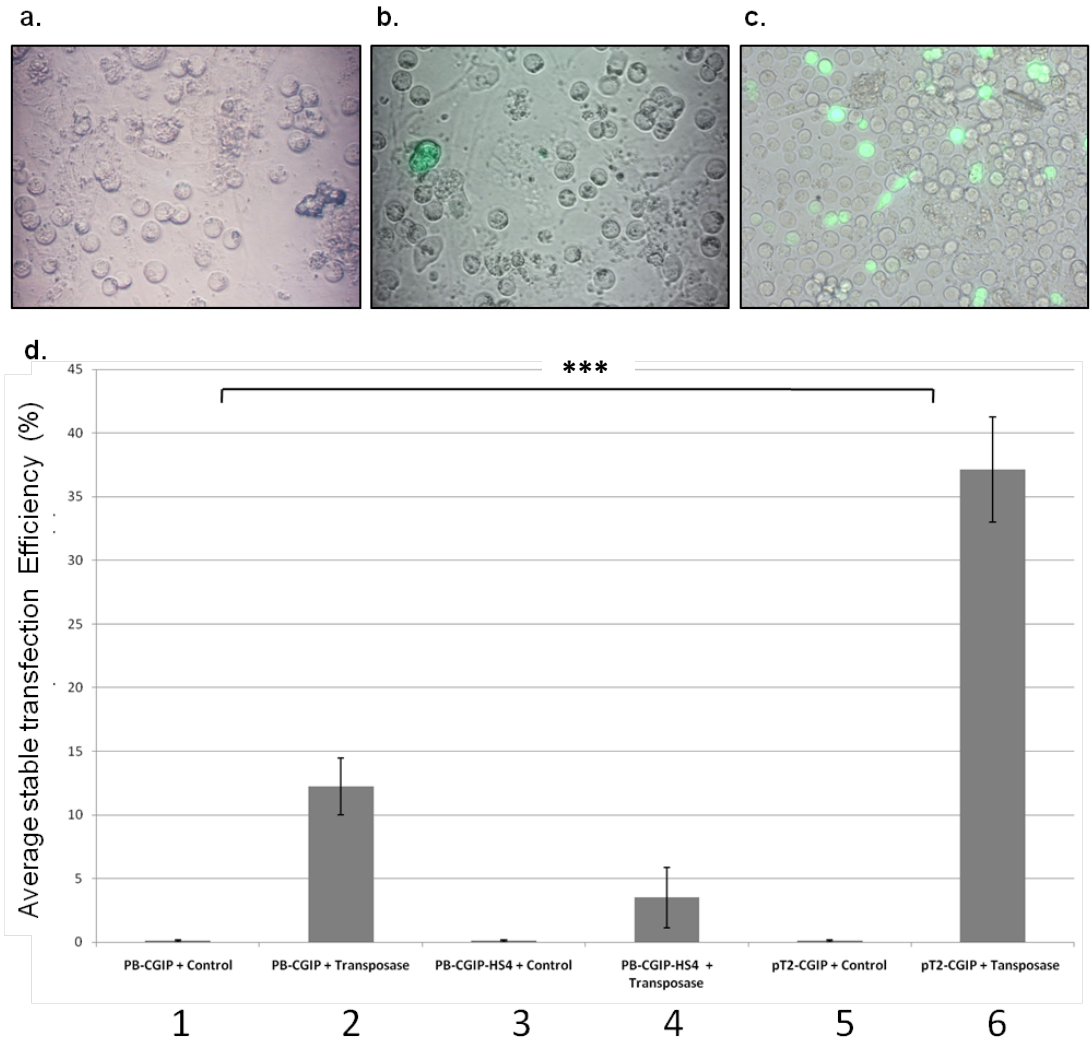


Figure 5.5 Stable transfection of chicken PGCs. Images of cultured chicken PGCs three weeks post transfection with (a) PB-CGIP without transposase (representative of any of the transposon-derived vectors transfected without transposase), (b) the piggyBac transposon vector PB-CGIP and transposase and (c) the *Tol2* transposon vector pTol2-CGIPwith transposase. (d) Direct comparison of integration rates of piggyBac and Tol2 transposons in primordial germ cells: 1. PB-CGIP minus transposase; 2. PB-CGIP plus transposase; 3. PB-CGIP-HS4 minus transposase; 4. PB-CGIP-HS4 plus transposase; 5. pT2-CGIP minus transposase and 6. pTol2-CGIP plus transposase. Data corrected for transfection efficiency and represents three independent experiments on two separate lines of primordial germ cells. Error bars, SEM. The integration rates are corrected for the initial rate of transfection. ***, P <0.001.

and the stable transfection efficiency of each transposon vector. Considering only the transposon vectors in the presence of transposase data standard posthoc Tukey pairwise comparison between revealed a significant difference between all three transposon vectors ($p<0.001$). Interestingly unlike what was observed in the DF1 cells the presence of the insulator sequences in the PB-CGIP-HS4 vector resulted in a stable tranfection rate that was significantly lower ($p=2e-16$) in comparison to that on the PB-CGIP vector when both were transfected with transposase. These results indicate that gene silencing is not occurring in the PGCs.

5.3.4 Analysis of stably transfected chicken primordial germ cells ability to transmit though the germ line

Transposon-modified primordial germ cells were assessed for their ability to form functional gametes. PGCs from cell line 08-08-09, were transfected (Materials and methods 2.7.6) with a piggyBac transposon carrying a reporter cassette consisting of a hybrid promoter CAGG, containing the CMV-IE enhancer fused to the chicken β-actin promoter and the first intron. The CAGG promoter was used to drive expression of a fluorescent reporter myrGFP, a myristoylated GFP that localises to the cell membrane. Fused to the fluorescent marker was an IRES (internal ribosomal entry site) and puromycin selectable marker (Figure 5.6c). This vector was created by digesting the PB-CGIP vector with Eco RI (Figure 5.6a) and inserting myrGFP isolated by digestion with Xho I and Not I (Figure 5.6b). Stably transfected cells were selected by adding puromycin at 1mg/ml to the culture medium. After selection only a few cells were present in the culture, all of which expressed the membrane bound GFP marker (Figure 5.7a). Before the cells were injected in to embryos they were grown in culture for a further 67 days to allow time for the cells to recover from selection and increase in number. At time of injection the PGCs were over 200 days in culture. Approximately 1000 PGCs were injected into wildtype embryos, approximately stage 16 HH (Materials and methods 2.7.6). Injected embryos were cultured to hatch using the surrogate shell method (Perry 1988)(Materials and methods 2.5.4; Figure 2.1).

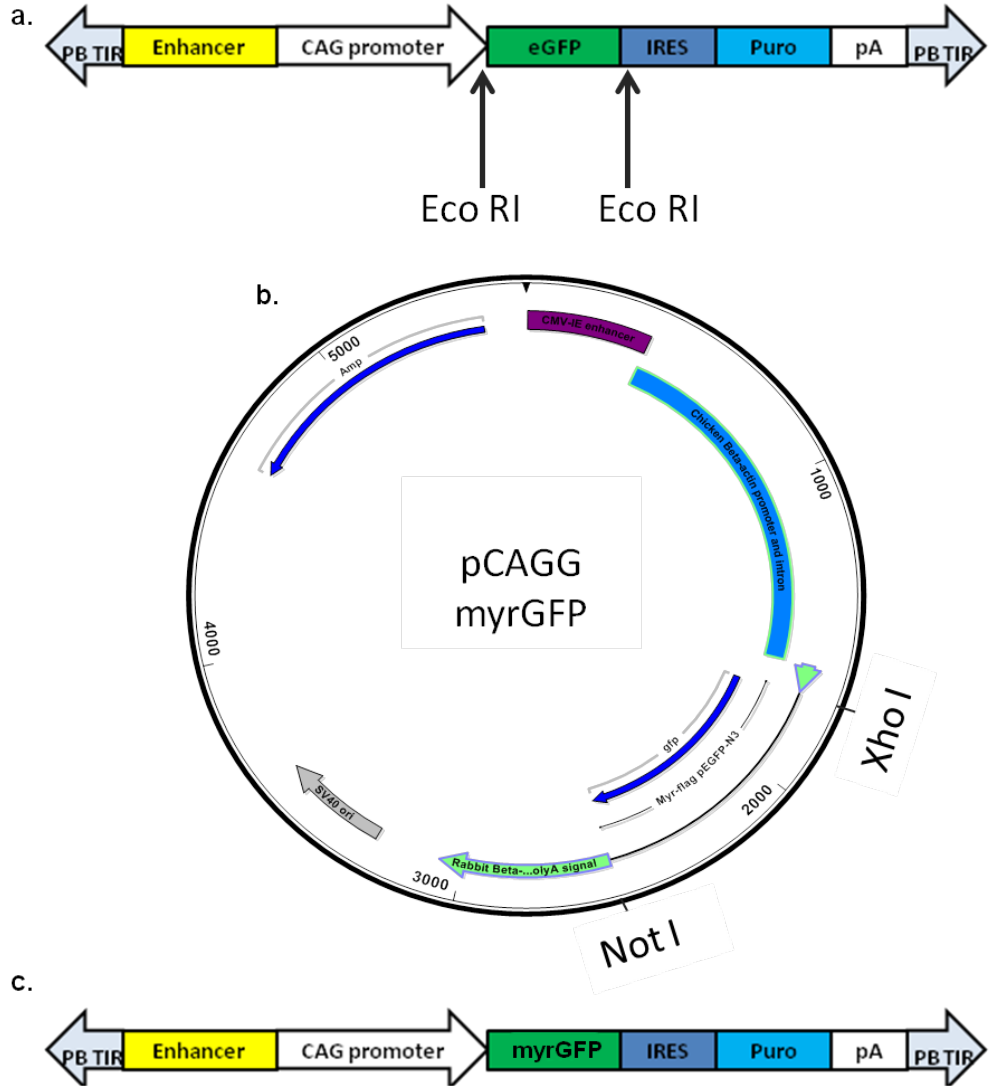


Figure 5.6 Plasmid maps for PB-CAGmyrGFP cloning. (a) Schematic of the PB-CGIP vector. (b) pCAGGmyrGFP plasmid map showing the restriction enzyme cutting sites used to excise the myrGFP (membrane expressing) gene. (c) Representation of the PB-CAGmyrGFP-IP vector. Inverted terminal repeats (Grey), internal ribosomal entry site (dark blue), puromycin selectable marker (light blue) and poly A tail (white)

A total of 16 embryos were injected and three survived to hatch. Several of the embryos that died during development were examined for the presence of GFP positive cells in the developing gonads at day five and ten of embryonic development (Figure 5.7b and c). Sections through the embryo show that far fewer than the 1000 injected cells have colonised the gonad. Few GFP-expressing cells were located in regions other that the region of gonadal development (Figure 5.7d) but it is unknown

what has happened to the other cells. W-PCR analysis of genomic DNA extracted from the chorioallantoic membrane (CAM) of the embryos surviving to hatch was used to sex the birds: two female (PGC4-1, PGC4-6) and one male (PGC4-10) (Figure 5.8a).

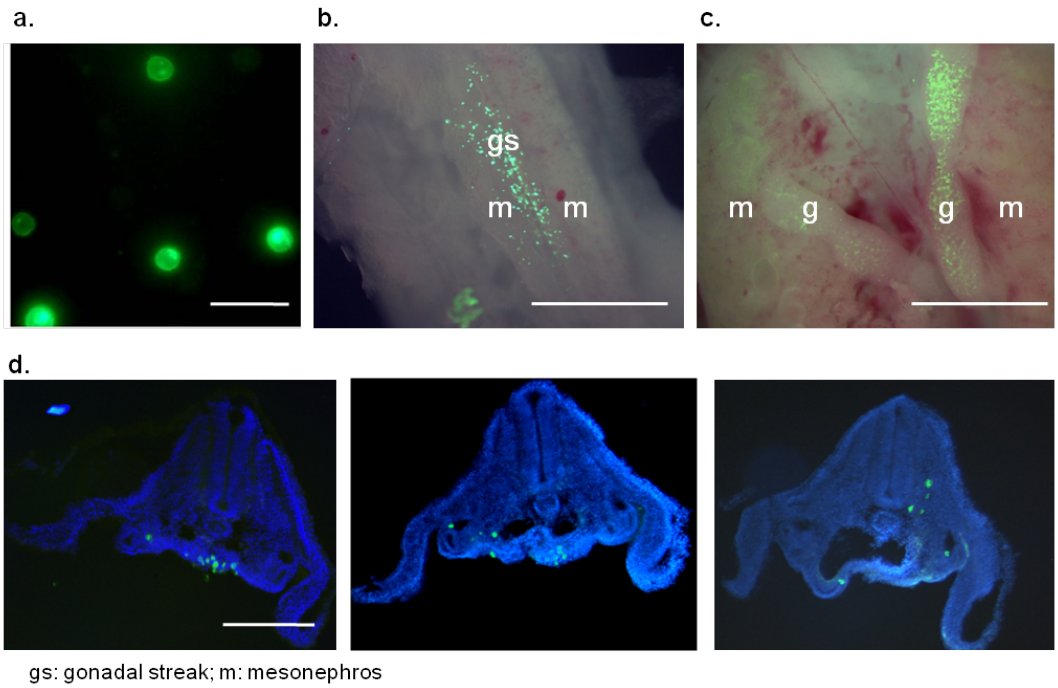


Figure 5.7 Membrane GFP+ PGC colonisation of the developing gonad. (a) Image of chicken PGCs stably transfected with membrane GFP piggyBac vector after puromycin selection. Bar 50µm. (b) GFP+ cellsand were observed to be present in the developing gonad at embryonic day 5 and (c) 10. Bar 50mm. (d) Transverse sections of the embryo show that GFP+ chicken PGCs are almost only present in the developing gonad.

The three potential germline chimeras were raised to sexual maturity and semen from the male bird was collected using abdominal massage and the genomic massage and the genomic DNA extracted. The genomic DNA was then assessed using semi-quantitative PCR to estimate presence of the transgene in the semen (Materials and methods 2.6.7). The semen was identified to be positive for the transgene that had been inserted in to the donor PGCs (Figure 5.8b) at an estimated frequency of 10%. As the GFP PGCs are heterozygous for the GFP allele and the semen is haploid, only half the actual transmission events would be observed. Based on this the germ line transmission was predicted to be approximately 20%. Due to the results in chapter 3 where none of the potentially germ line chimeric females transmitted the female

birds PGC4-1 and PGC4-6 were not assessed for transmission. Cockerel, PGC4-10 was crossed with stock hens and the hatchlings screened for GFP fluorescence to identify hatchlings deriving from the donor PGCs. Examination of 470 hatchlings produced only one GFP expressing bird. The corrected efficiency of germ line transmission for PGC4-10 was 0.4%. Examination of the testes from PGC4-10, post mortem showed the GFP^+ cells were present and that the amount of fluorescent tissue was comparable to the PCR estimate (Figure 5.8d).

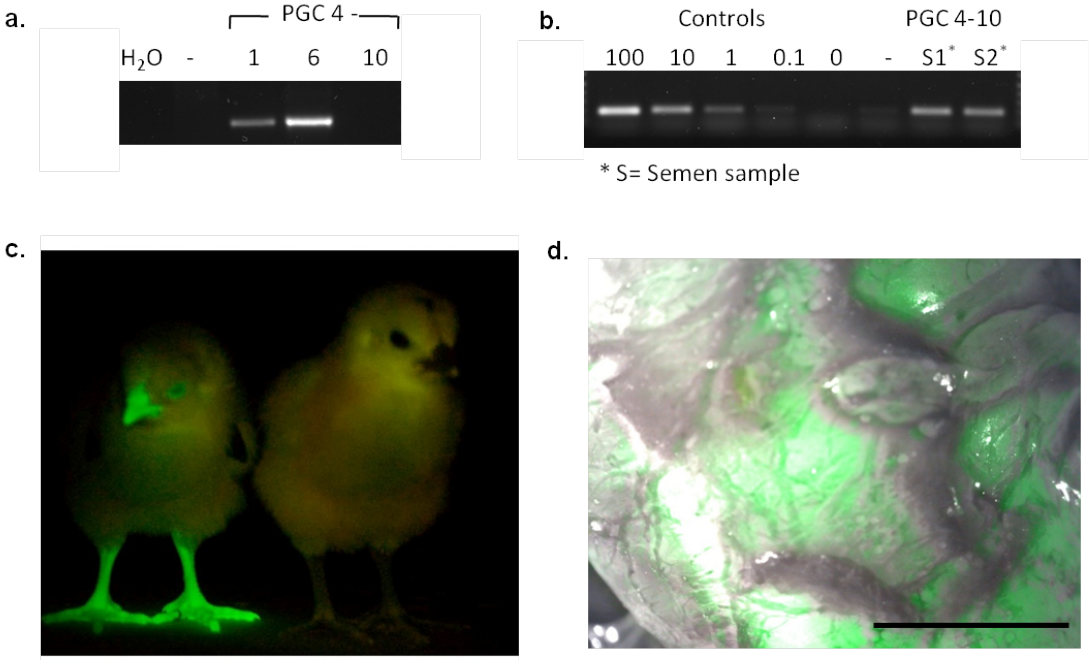


Figure 5.8 Genetically modified PGCs can form functional gametes. (a) W-PCR of the three embryos injected with the transposon modified PGCs that survived to hatch. (b) The male bird, PGC4-10 was raised to sexual maturity. PCR was carried out on 50ng of genomic DNA extracted from the semen. Copy number controls were set up in parallel using non-transgenic DNA spiked with varying amounts of vector DNA plasmid producing concentrations equivalent to one copy of the transgene, per genome (100%), per ten genomes (10%), per 100 genomes (1%) and per 1000 genomes (0.1%). (c) The G1 offspring from bird PGC4-10, the bird on the left is GFP^+ was produced from a donor germ cell whist the bird on the right is a non-transgenic hatch mate produced from PGC4-10's own germ cells. (d) Image of testes from PGC4-10 showing GFP expressing tissue. Bar 25mm.

5.3.5 Analysis of the putative transposon integration site

To detect the number of integration sites resulting from transfection of chicken PGCs that went on to produce a transgenic bird, Southern blot analysis was carried out. It

was also important to confirm that integration of the transgene had been transposase-mediated and into which regions of the genome these transposition events had occurred. To do this inverse PCR analysis was carried out.

5.3.5.1 Southern blot analysis of transposon vector integrations in G0 and G1 birds

Genomic DNA was prepared from blood from a control hen, semen from the G0 cockerel PGC4-10 and blood from G1 bird PGC4-10:398. To produce junction fragments for the 5' and 3' end of the integration site the DNA was digested using enzymes that cut once within the transgene (Figure 5.9a). DNA was digested with Spe I to produce the 5' junction fragment and Pst I to produce the 3' fragment was carried out. The DN samples were also double digested with both Spe I and Pst I. A probe to detect the 1kb GFP gene was made by digesting the PB-myrCGIP vector with Not I and Age I (Figure 5.9a). Although the quality of the blot shown in figure 5.9b is poor it is possible to see that no transgene was detected in the controls. 5' and 3' junction fragments, of the expected sizes 6kb and 4.5kb respectively were detected in both the DNA from PGC4-10 semen (marked with red dots) and PGC4-10:398 blood. The fragment produced by the double digestion of the DNA samples produced a fragment of the expected 4kb size corresponding to the size of the reporter cassette. One insertion site was detected in the genomic DNA extracted from the semen (marked by red dots). However the poor quality of the blot may also be masking the observation of other insertion sites.

5.3.5.2 Inverse PCR identification of transposon-mediated transgene integration

The 5' (left side) junction site between the integrated transposon and chromosomal DNA in PGC4-10:398 was isolated using inverse PCR and the PCR product sequenced. Part of the putative chromosomal DNA sequence was shown to be identical to a region on chromosome 22 within the first intron of the PARD6B gene (Figure 5.10). The rest of the sequence was identified to be identical to then 3' region of the vector. This analysis confirmed that the 3' region of the transposon-vector was

intact. A TTAA repeat, specific to piggyBac integration, was located at the site in which the 3' end of the transposon vector had integrated (Figure 5.11, top). Sequencing of a PCR product generated using a primer located in the 5' region of genomic DNA and a vector-specific 3' primer (Table 2.2) validated the result from the initial PCR analysis. The 3' (right side) junction between the 5' end of the vector and the chromosomal DNA was not isolated using inverse PCR. Using a primer located in the 3' region of genomic DNA and a vector-specific 5' primer a PCR product was generated and sequenced. The sequence matched the 5' end of the vector and the chromosomal DNA sequence contiguous with the site of integration in PARD6B on chromosome 20 (Figure 5.11 bottom). A TTAA repeat was also identified at the site where the 5' end of the vector had integrated (Figure 5.11 bottom).

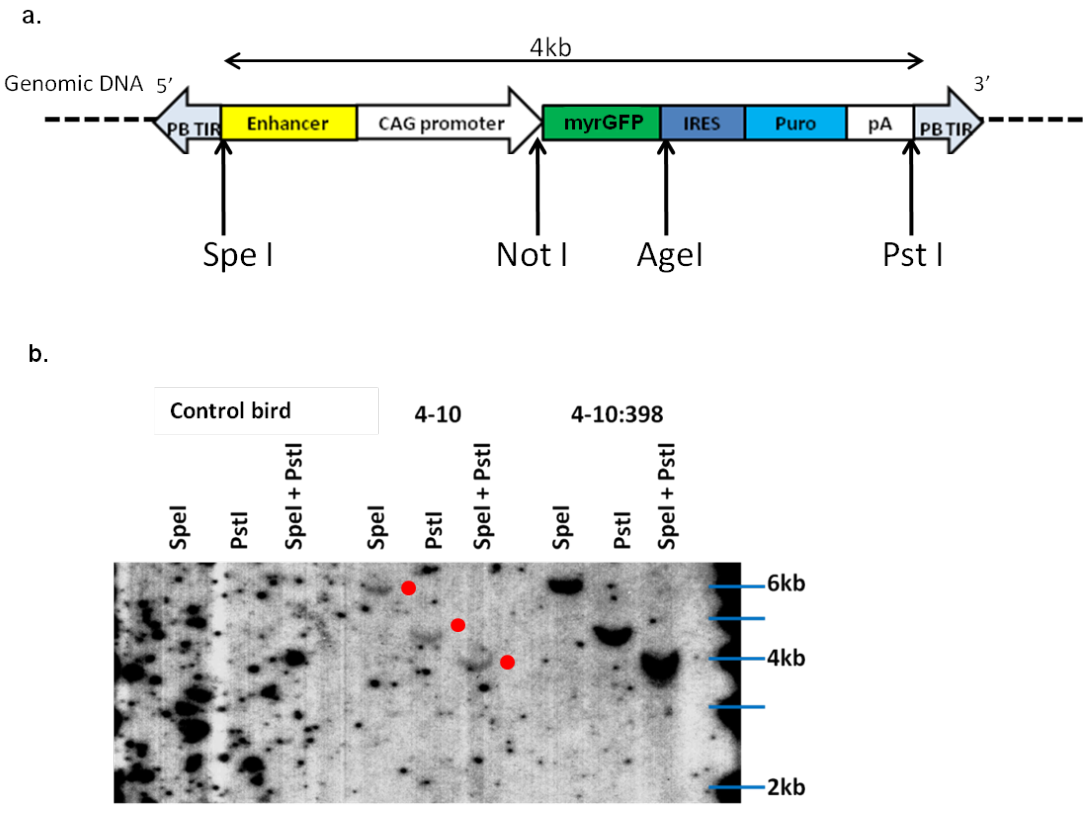


Figure 5.9 Analysis of genomic integration of piggyBac flanked gene cassettes. (a) Representation of the transgene cassette transposed into genomic DNA with restriction enzyme sites labelled. (b) Southern blot of genomic DNAs prepared from WT blood (Control bird), bird 4-10 semen and bird 4-10:398 blood samples, digested with SpeI, PstI or SpeI/PstI and hybridised with a probe for the GFP coding sequence. Inverted terminal repeats (Grey), internal ribosomal entry site (dark blue), puromycin selectable marker (light blue) and poly A tail (white)

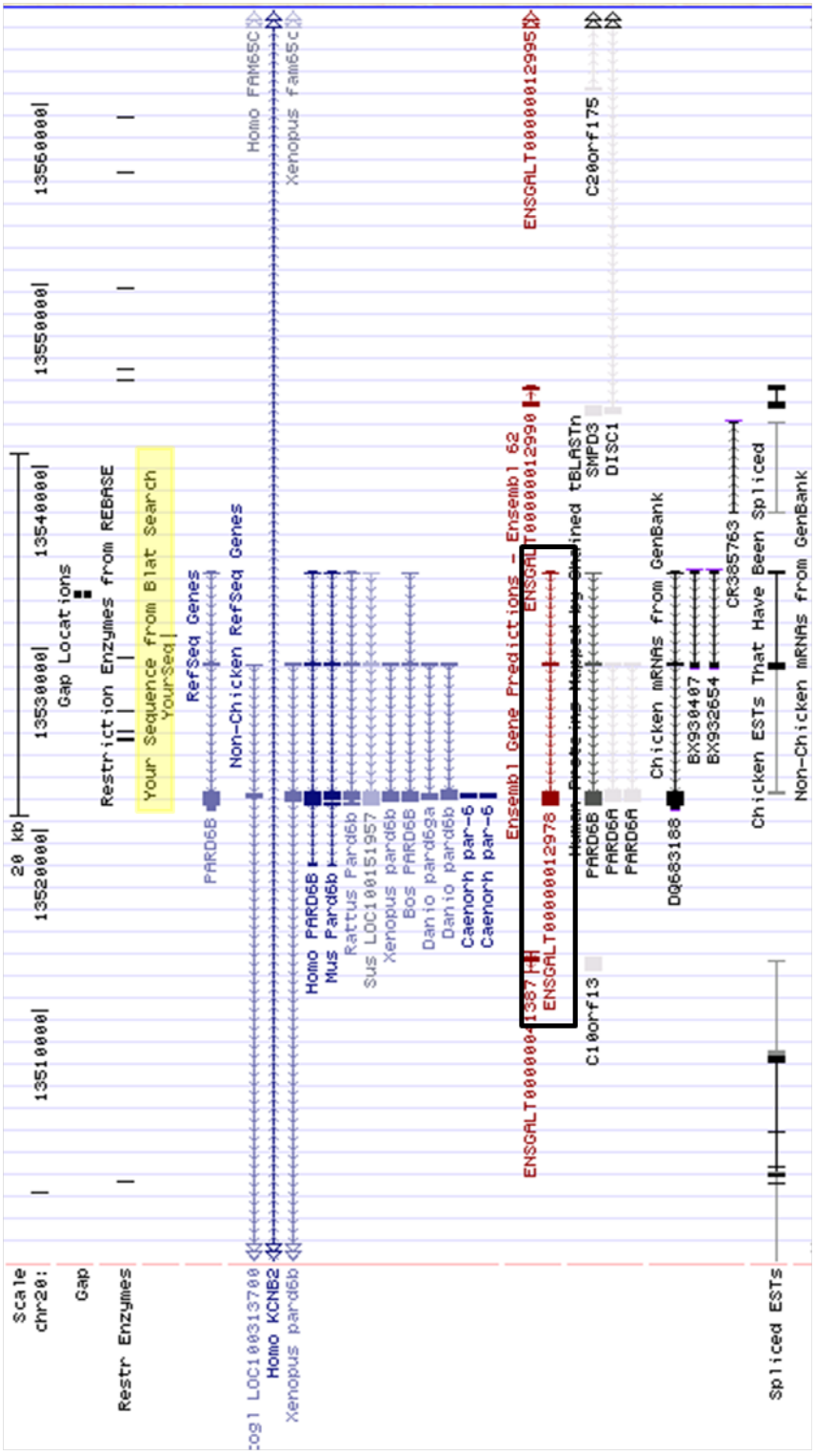


Figure 5.10 Genomic integration of GFP reporter mapped to PARD6B gene on chromosome 20. Result from BLAT analysis (UCSC genome browser) of inverse PCR product sequence shows integration of the transgene reprter cassette in in the first intron of the PARD6B on chromosome 20.

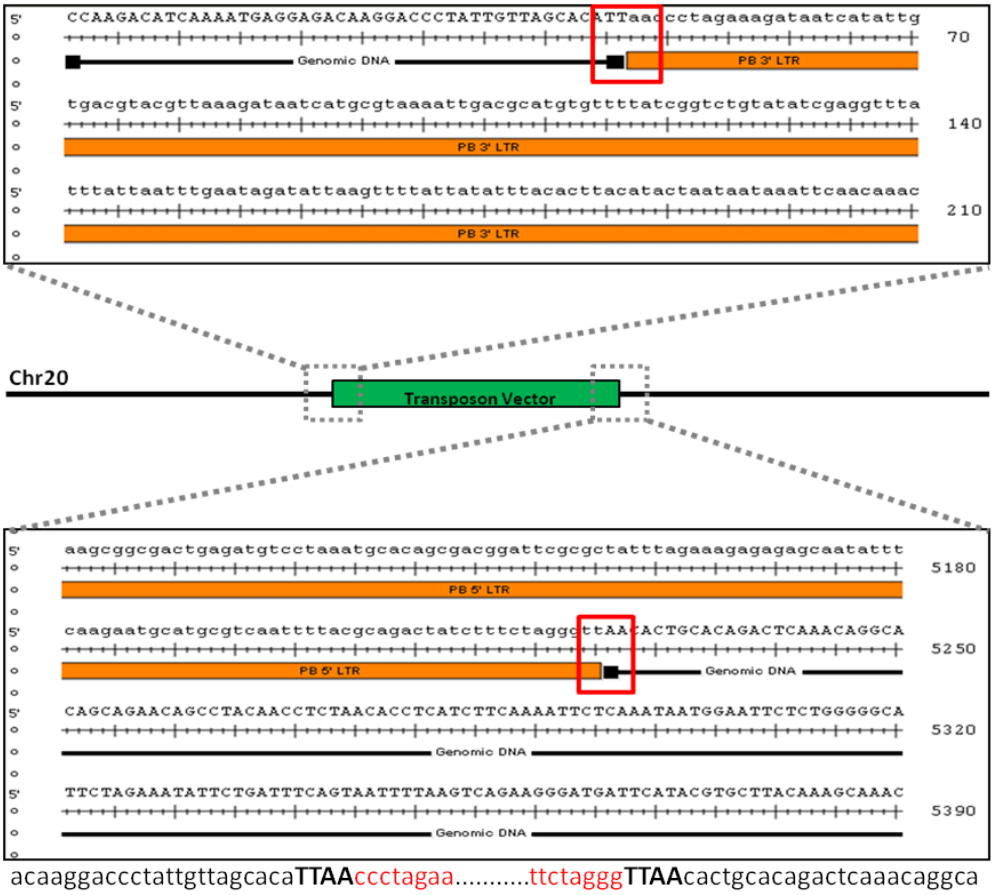


Figure 5.11 Sequence alignment of GFP reporter cassette. Sequence from PCR products produced for the 5' and 3' ends of the integration aligned to PB-CAGmyrIP vector show the TTAA repeat synonymous with piggyBac transposase-mediated genomic integration. LTR=ITR, inverted terminal repeat.

5.4 DISCUSSION

Van de Lavoir *et al*. (2006) established a protocol for the sustained growth of chicken PGCs. This provides a valuable cell based method for the production of transgenic birds. Initial attempts to manipulate the PGCs were unsuccessful and the lack of transgenesis was attributed to gene silencing of expression of the GFP marker used. Using insulator sequences isolated from the chicken β globin gene transgenesis was achieved using both DNA vectors and the bacteriophage ΦC31 integrase (van de Lavoir *et al*. 2006, Leighton *et al*. 2008) but at low efficiency. In an attempt to improve the efficiency with which the PGCs could be modified, vectors derived from the transposons Tol2 and piggyBac were assessed here for efficiency in modification of chicken PGCs. Vectors were made both with and without insulator sequences in light of the reported effects of gene silencing.

Transposon-derived vectors were chosen as they offer significant potential for the trangenesis of chicken PGC and have been shown to be effective vectors for use in the modification of species including fish, mouse, human and chicken (Ivics *et al*. 1997; Kawakami *et al*. 2000; Handler 2002; Ding *et al*. 2005; Hamlet *et al*. 2006; Sato *et al*. 2007; Chen *et al*. 2010). Although the germ lineage was modified in these animals, as evidenced by the germ line transmission of the transposons, it has not been confirmed whether or not the transposons were functional in the early germ cell lineage, such as the PGCs of the founder animals. This makes the work presented here valuable in determining if transposons can be used for germ cell research.

In this chapter it has been shown that not only are transposon-derived vectors functional in cultured chicken cell lines but that they can be used directly to modify chicken PGCs efficiently. Interestingly the results outlined here do not indicate that gene silencing is having an effect on the expression of the genes that have been inserted into the DNA of the PGCs using the vectors derived from either the piggyBac or Tol2 transposable elements.

5.4.1 Transposon-mediated transgenesis of chicken DF1 cells

Chicken fibroblast cell line DF-1 was transfected with vectors derived from the piggyBac or *Tol2* transposons carrying a GFP reporter gene. When each vector was transfected in the presence of transposase, the frequency of transgenesis occurred 5 (PiggyBac) and 10 (Tol2) times more frequently than was observed in controls (Figure 5.3a). Comparison of the piggyBac vectors with and without the insulator sequences showed significant difference in stable transfection rates (Figure 5.3b). These results indicated that transcriptional silencing may have been occurring in the DF1 cells. It was also observed that on average the *Tol2* vector system was more efficient that the piggyBac system at mediating transgene integration into to the chicken genome. However, since this work was carried out a hyperactive piggyBac transposase has been developed that has been shown to improve integration rates by as much as 9-fold in mouse cells (Yusa *et al*. 2011).

5.4.2 Transposon-mediated transgenesis of chicken PGCs

All three transposon-derived vectors were used in the transgenesis of established chicken PGC cultures. It was shown that as in the DF1 cells, the *Tol2*-derived vector was more efficient than the piggyBac derived vectors, 36.5% in comparison to 4% and 12.5%. The observation of DNA vector silencing that has been documented in cultured chicken PGCs transfected with DNA plasmid without insulator elements (van de Lavoir *et al*. 2006) and shown to be potentially occurring in the DF1 cells was not observed in the chicken PGCs. This was concluded as the number of PGCs that were stably transfected using the piggyBac vector with insulator sequences flanking the reporter cassette; PB-CGIP-HS4 produced significantly fewer ($P<0.001$) stably transfected cells than the piggyBac vector without the HS4 sequences; PB-CGIP. Overall the stable transfection rates presented here were higher than any of the previously published data using DNA plasmids or the phiC31 integrase in the presence of insulator elements (Van de Lavoir *et al*. 2006, Leighton *et al*. 2008). This indicates that vectors derived from either the piggyBac or *Tol2* transposons are more effective than either or the two previously used methods for the manipulation of the chicken genome in cultured PGCs.

5.4.3 Stably transfected chicken primordial germ cells can generate transgenic birds

The production of genetically manipulated chicken PGCs that retain their ability to form function gametes will provide an invaluable tool for the development of transgenic chicken lines. To demonstrate that tranposon-mediated integration does not inhibit germ line transmission a piggyBac vector carrying a membrane GFP reporter and puromycin selectable marker was used to stably transfect chicken PGCs (Figure 5.5a).

Chicken PGCs that were stably transfected were selected for using puromycin. After selection the cell number was allowed to recover and by the time cells were injected in to host embryos the PGCs had been maintained in culture for over 200 days. The cells were injected in to embryos and shown to colonise the gonad of embryos at day five and ten of development (Figure 5.5b and c). On examination of the embryos GFP positive cells were situated in the region of gonad development. However, much fewer than expected cells were observed in the cross-sections of the embryos compared to the number injected.

To demonstrate that the manipulated PGCs were still able to form functional gametes they were injected into embryos that were taken to hatch. One male and two female potentially chimeric birds survived to hatch. All three were raised to sexual maturity. PCR transgene in the semen (Figure 5.7b) identified that 10% of the DNA sample was positive for the transgene indicating an expected transmission rate of 20%. Given the results observed in chapter three only the chimeric male was crossed to wildtype stock hens. One GFP positive bird was produced from 457 chicks hatched, resulting in a transmission rate of 0.4%. This was much lower than the 20% transmission frequency that had been predicted from the semen sample. In both the van de Lavoir *et al*. (2006) and Choi *et al*. (2010) papers transmission of modified PGCs was achieved using cultures that had been maintained in culture for up to 136 days. Van de Lavoir *et al*. (2006) demonstrated 49% transmission of the PGC derived phenotype. The cell used in the experiment outline here had been in culture

for over 200 days. Length of time in culture could be affecting the transmission frequency, much like what was observed in the chicken ES cell culture. Without more data no real conclusion as to why such a low transmission rate was observed can be made.

Interestingly within the laboratory, membrane GFP transgenic birds have been produced using lentiviral vectors. Only one viral insertion was identified and the transgenic birds are diminished in size compared to wildtypes. It should also be noted that the one GFP^+ bird that was produced from the membrane GFP PGCs was not robust and died unexpectedly at around 16 weeks. As well as these results membrane cherry transgenic birds have also been produced using the lentiviral vector technique. As with the membrane GFP birds they were far less robust than their wildtype counter parts and none survived to sexual maturity. Taking all of these factors into consideration it is hypothesised that membrane expressed fluorescent proteins may be causing a reduction in production of viable sperm from the membrane GFP PGCs that were used here. This hypothesis is supported by the level of GFP expression observed in the testes of PGC4-10 post mortem that were representative of what had been shown by PCR (Figure 5.8). It is also worth noting that karyotype analysis was not completed given the difficulties involved with identifying the numerous microchromosomes of the chicken karyotype.

5.4.4 Identification of the transposon vector integration site

Transposon-mediated modification can result in multiple integration sites per genome. To verify the number of transgene integrations Southern blot analysis of the genomic DNA from both the semen of the G0 bird and the transgenic hatchling was carried out. Using a probe specified to the GFP transgene junction fragments for both the 5' and 3' ends of the transgene were identified. In total, one integration site was detected suggesting that only one integration event had occurred. However the poor quality of the blot has to be considered and had this been improved other integration sites may have been detected from analysis of the semen sample. It should also be considered that after puromycin selection only a few stably transfected PGCs were

left in the cultures. These cells were allowed to proliferate to a suitable population prior to injection and it may be that PGCs carrying the detected integration site proliferated more readily than PGCs carrying a different integration. This would have resulted in a greater number of spermatozoa positive for this integration and increased possibility of it being transmitted. As the transmitted insertion site is itself only identified at low levels within the semen sample, any other integration sites that were present at lower levels would not have been detected. The result could also be attributed to the puromycin selection. If it was too stringent then cells with the transgene integrated at a different locus may have been killed off.

Integration of transgenes into the genome can occur via random integration, the basis for stable transfection. This was observed in the DF1 cells where stable transfection of the transgene occurred in the absence of transposase expression. However in the chicken PGCs stably transfected cells were only produced when the cells were transfected with the transposon-derived vectors and the appropriate transposase. To confirm that transposase-mediated integration had occurred inverse PCR was performed and the site of integration sequenced. The site of transgene integration mapped to the first intron of the PARD6 gene on chromosome 20 (Figure 5.10). This fits with what is known about the preference for piggyBac transposons to integrate into intronic regions (Wilson *et al* 2007). This was further confirmed by the presence of TTAA integration sites that are specific to piggyBac insertions at the either side of the integration site (Figure 5.11).

5.5 CONCLUSIONS

From these results it can be concluded that vectors derived from transposable elements can be used efficiently to modify cultured chicken PGCs. The rates of stable transfection, presumed integration far exceed those outlined in previously published work (van de Lavoir *et al*. 2006; Leighton *et al*. 2006) It has been demonstrated here that transposon mediated modification does not prevent cells from forming functional gametes. However the transmission rate observed was lower than predicted based on the PCR analysis of the semen DNA. Not enough data is available to make any conclusions with regards to the low transmission rates but as discussed other observations within the laboratory suggest that the membrane GFP expression may be toxic to the cells resulting in a lack of functional sperm. Despite low transmission rates the results presented here show significant advancement in the ability to genetically modify cultured chicken PGCs for the future production of transgenic chicken lines.

CHAPTER 6: GENERAL DISCUSSION

6.1 INTRODUCTION

The objectives of the experiments described in this thesis were to establish cultures of chicken primordial germ cells for utilisation as a cell based method for the genetic manipulation of the chicken genome and as a tool for the investigation of early germ cell development. It was therefore necessary to recapitulate the previously published culture method in order to establish chicken PGC lines. This proved difficult and required analysis of the culture method and highlighted the requirement for an improved culture medium. Once the cultures were established it was necessary to confirm that the cells still had all the characteristics of PGCs including capability of forming functional gametes.

6.1.1 Establishing cultures of chicken PGCs

Chicken PGCs were isolated from chicken embryonic blood containing migratory PGCs. *In vitro* culture of the PGCs was for some time unsuccessful using the van de Lavoir (2006) method and alterations to the culture medium were tested. This involved assessing several commercially available FBS and chicken sera, addition of recombinant growth factors hFGF2 and mSCF and assessment of the cytokine mSDF1.

First it was established that all blood samples used in the attempted establishment of chicken PGC cultures contained circulating PGCs. By transferring blood from a GFP expressing stage 15 HH embryo directly into same stage wild type embryos it was established that all initiated cultures had the potential to form established cells lines. However this was found not to be the case. When establishing cultures and assessing culture conditions a successful isolation of PGCs from embryonic blood was the presence of 100 or more PGCs in a culture three weeks post aspiration of embryonic blood. Using the van de Lavoir *et al*. (2006) method 94 cultures were initiated none of which resulted in an established PGC line.

To assess why cultures were not being successfully established commercially available sera were assessed for their ability to support the propagation of PGCs *in vitro*. Sera contain unknown levels of growth factors and cytokines that can have either stimulatory or inhibitory effects on cell culture. FBS is used in maintenance of mouse and chicken ES cells (Evans and Kaufmann 1981; Pain *et al*. 1996) and has been shown to important in the maintenance and proliferation of chicken PGCs (van de Lavoir *et al*. 2006; Tang *et al* 2007). 240 separate blood samples were used to test four bovine and three chicken sera. It was established that PAA laboratories ES cell tested FBS and either Sigma's or Biosera's chicken serum best supported PGC outgrowth and were used for all further PGC culture.

After establishing the most appropriate sera to use the addition of recombinant growth factors to the culture medium was assessed. Recombinant FGF2 from human and SCF are components of the van de Lavoir method, however the species of SCF is not stated in the paper. As the concentration used is specified it was assumed that a commercially available growth factor had been used. It was decided for the experiments presented in chapter 3 that murine SCF (R&D systems) would be the most appropriate growth factor to use. Cell cultures were initiated from embryonic blood in basic culture medium (Materials and methods 2.1.6) plus or minus hFGF2, mSCF or FGF2 and mSCF. The results showed that addition of mSCF did not improve the efficiency with which chicken PGC cultures could be established and although not statistically proven indicated that the presence of mSCF might have had an inhibitory effect. This observation is supported by the work of Choi *et al* (2010) who demonstrated that proliferation of chicken PGCs was lower in hSCF and hFGF2 treated cultures than cultures where only hFGF2 was added. In the culture conditions used here were only hFGF2 was added significantly more ($P<0.05$) cultures were shown to result in successful isolation of chicken PGCs from embryonic blood. This was in comparison to cultures where; neither hFGF2 nor mSCF were added, mSCF was added alone or where hFGF2 and mSCF were added. These results once again fit with the observations of Choi *et al* (2010) where it was demonstrated that addition of hFGF2 only to the culture medium caused the biggest observed increase in PGC

proliferation compared to culture medium where other growth factors such as hSCF and hLIF were also added. These results are similar to what has been observed in mouse PGC cultures where it has been shown that addition of FGF2 is essential for isolation (Resnick *et al*. 1992; Matsui *et al* 1992). However unlike mouse PGCs, the chicken PGCs cultured did not differentiated into EG cells in the presence of FGF2 after several days in culture.

SDF1 is not a component of the van de Lavoir culture medium but is required for PGC migration, proliferation and survival in many vertebrates (Knaut *et al*. 2002; Molyneaux 2003; Takeuchi *et al*. 2009) and in PGC migration in the chicken embryo (Stebler *et al*. 2004). PCR analysis showed that chicken PGCs expressed the mSDF1 receptors, CXCR4 and CXCR7. When mSDF1 was added to the medium the number of PGCs in culture increased significantly ($p<0.02$) over the course of two weeks in comparison to the cells cultured for the same time period in van de Lavoir culture conditions. This suggests that as well as the documented role in migration of chicken PGCs that mSDF1 may also promote cellular proliferation and survival.

To show that mSDF1 was inducing cellular proliferation through interaction with the CXCR4 and not the CXCR7 receptor cells were cultured in the presence of the inhibitor AMD3100. AMD3100 blocks the interaction of mSDF1 and CXCR4. The results showed cells cultured in the presence of AMD3100 and added mSDF1 did not proliferate at the same rate as the control cells cultured in the van de Lavoir *et al* (2006) culture conditions with added mSDF1. These results indicated that in chicken PGCs, SDF1 induces proliferating via interaction with the CXCR4 receptor and not CXCR7. When AMD3100 was added to cells grown without added mSDF1 and compared to control cultures, fewer cells were observed in the AMD3100 treated cultures. This indicated that SDF1 was already present in the culture medium and as this contains chicken serum, FBS and conditioned medium the source of SDF1 could be any one or all of these components.

Addition of SDF1 to culture medium was shown to improve isolation of chicken PGCs from embryonic blood. The biggest improvement in isolation efficiency was observed when SDF1 and hFGF2 were both added to the culture medium. When SDF1 was added at half the original concentration used (25ng/ml to 12.5ng/ml) this improved isolation efficiency in SDF1 only treated cultures. The improvements in isolation frequency linked to SDF1 added to medium was coupled with change in the PGC morphology, cells formed large semi-adherent colonies. A biological reasoning for this was linked to the interaction of SDF1 and its second receptor CXCR7. The function of CXCR7 is to sequester SDF1 when it is present at high levels *in vivo* (Mahabaleshwar *et al*. 2008; Naumann *et al*. 2010). It is perhaps possible that due to the role SDF1 plays in migration *in vivo* that surface bound SDF1 resulted in an attraction between the cells. If CXCR7 binds a different site on the SDF1 than CXCR4 this may result in competition between receptors on two cells for the same SDF1 molecule. This could result in a bringing together of cells to form floating colonies.

Seven chicken PGC lines were established all of were identifiable by their characteristically round shape, large nucleus, granulated cytoplasm and non-adherence to the feeder layer. Gene expression analysis was used to further characterise the isolated cells. Durcova-Hills *et al* (2008) had showed that mouse EG cells expressed several marker of pluripotency that are used to define an ES cell, *nanog*, *Sox2*, *Oct3/4*, *c-Myc* and *Klf4*. They also demonstrated that one of the differences between mouse EG cells and PGCs was that mouse PGCs express *nanog*, *Sox2* and *Oct3/4* but not *c-Myc* and *Klf4* and suggested that this difference in gene expression accounts for PGC restriction to the germ line. In contrast when chicken PGCs from the lines established during this project were analysed and compared to chicken ES cells for expression of these genes it was shown that both expressed all five. One possible biological basis for the differences in gene expression observed between mouse and chicken PGCs may be related to the way in which the germ line is formed. The mouse germline has been shown to form in the absence of maternally inherited cytoplasm by induction mediated by signals from surrounding somatic

tissue such as bone morphogenic proteins (BMPs) (Tam and Zhou 1996; Lawson *et al*. 1999; Ying *et al*. 2000; Ying and Zhao 2001). In the chicken the germline is determined by inheritance of cytoplasmically localised proteins and RNA laid down in the oocyte. To better understand formation of the chicken germline it would be beneficial to compare these results with gene expression from other species such as zebrafish and *Xenopus* whose germ line is determined by maternally inherited factors.

The established chicken PGC lines were expanded from only a few initial cells to cultures of more than 100,000 and were propagated for more than 200 days. Interestingly all the cultures were shown by W-PCR to be male. Although female chicken PGCs have been successfully cultured by both van de Lavoir (2006) and Choi *et al* (2010), maintenance of female PGCs *in vitro* has been significantly less successful than the derivation of male cultures. These differences could result from variations in hormone level or pH in the culture medium that are more conducive to the survival of male PGCs. Work carried out by Zhao *et al*. (2010) where in a series of reciprocal sex (male to female; female to male) transplantation experiments it was shown that cells of the germ lineage from different sexes are inherently different demonstrated by the exclusion of donor cells from functional structures.

The PGC cultures were established for a number of purposes including utilisation as a tool for the genetic manipulation of the chicken genome. To show that these cells were suitable for this purpose, their ability to form functional gametes after propagation in culture had to be demonstrated. Two of the PGC lines, 10-08-09 and 06-10-10 were GFP expressing and were shown to migrate to and colonise the gonad. Cells from PGC line 10-08-09 were injected into host embryos that were taken to hatch. Seven male germline chimeras were produced all of which were identified to be positive for the GFP transgene and the three cockerels tested all fathered chicks carrying the GFP transgene, confirming that the PGCs were able to form functional gametes.

Along with the male germline chimeras, three potential germline chimeric female birds were produced. All three hens were tested but no transgenic chicks were hatched from a total of 188 embryos. This was supported by van de Lavoir *et al* (2006) who showed that in chicken, male PGCs are unable to form functional gametes when transplanted into a female host embryo. These results indicate that male chicken PGCs are unable to form functional oocytes in a female host. In contrast, it was shown in mouse that whilst female PGCs do not form functional gametes in male hosts (Plamer 1991), male PGCs enter meiosis and differentiate in accordance with female host embryo development (Kocer *et al*. 2009). Furthermore male PGCs were able to form functional oocytes in female mice (Ford *et al*. 1975). These results indicate sex-specific differences in chicken germ cells. Interestingly female mouse PGCs lack a Y chromosome and do not form functional gametes in a male host and male chicken PGCs lack a W chromosome and are unable to form functional oocytes. So perhaps chromosome complement is having a biological effect on these cells ability to form functional gametes in opposite sex hosts.

6.1.2 Analysis of signalling pathways essential for PGC survival *in vitro*

The difficulties encountered in reproducing the van de Lavoir method highlighted the importance of gaining a better understanding of the requirements for chicken PGC survival in culture. A better understanding of germ cell biology is important to the development of a more robust system for the culture and propagation of PGCs *in vitro*. The aim of the research described in chapter 4 was to examine three signalling pathways, PI3K/AKT, MEK/ERK and JAK/STAT and determine what components of the culture medium induced their activation and the requirement of the individual pathways in the propagations of chicken PGCs in culture.

To test the requirement of the individual pathways for the propagation of the PGCs in cultures small molecule inhibitors to block signalling through each pathway were used. Three different cell lines were assayed three to six times in individual experiments. When the inhibitor LY294002, which blocks signalling through PI3K (De Miguel *et al*. 2002) was added to cultures the number of cells in the well did not

increase over the course of the experiment and by the end of the seven days there were significantly more cells in the control well ($P<0.01$). The same observation was made between control cells and cells treated with the MEK inhibitor PD0325901 (De Miguel *et al*. 2002; Takeuchi *et al*. 2005) over the same time course. PD0325901 inhibits signalling through the MEK/ERK pathway and this was confirmed by western blot analysis, which showed that phosphorylated ERK1/2 was absent from cells treated with the inhibitor. This confirmed a complete down regulation of the pathway as a result of PD0325901 treatment. Analysis of the JAK/STAT pathways function in cell proliferation was shown to be the same as both the PI3K/AKT and MEK/ERK pathways. When cells were cultured in the presence of 420099 the cell number after seven days was significantly lower ($p<0.01$) than in controls. These results indicated that all three pathways were essential for the propagation of chicken PGCs *in vitro*.

To define how these pathways are stimulated in chicken PGCs in culture, the van de Lavoir culture medium was dissected and the individual components BRL-conditioned medium, chicken sera, FBS, recombinant growth factors FGF2, SCF, LIF and IGF1 were investigated. Western blot analysis was used to detect activation of the pathways in cultures of chicken PGCs grown in starvation medium for four hours and then cultured with each of the seven components individually.

When starved chicken PGCs were cultured in the presence of hFGF2 only the MEK/ERK pathway was activated shown by detection of phosphorylated ERK in the total cellular protein. This result was unsurprising as FGF2 is well documented to induce the MEK/ERK pathway (Katz *et al* 2007). FGF2 amino acid sequence is conserved between chicken and humans and this result and the improvement in chicken PGC isolation from embryonic blood observed by addition of hFGF2 to culture medium indicate that FGF2 from human is functional in chicken cells. Choi *et al* (2010) also showed that chicken PGC proliferation was increased when hFGF was added to cultures. Interestingly FGF2 has also been shown to activate the PI3K/AKT pathway in mouse EG cells (Kimura *et al*. 2007). The lack of FGF2

induced PI3K/AKT activation may be due to a control mechanism active within the PGCs inhibiting chicken PGC dedifferentiation.

SCF induced signalling promotes proliferation and survival in the culture of mouse PGCs (Dolci *et al*. 1991; Godin *et al*. 1991; Matsui *et al*. 1991; Manova *et al*. 1992; Pesce *et al*. 1993). However as shown in chapter 3 and reported by Choi *et al* (2010) chicken PGCs can be propagated without the addition of mouse or human SCF to culture medium. SCF\c-Kit interaction can induce the PI3K/AKT (Sette *et al*. 2000; De Felici 2000; Liu *et al*. 2007) and MEK/ERK (Dolci *et al*. 2001) pathways. Based on sequence homology it was predicted that chicken and mouse SCF would not be functionally conserved between species. This was confirmed by the western blot analysis, which showed that only the SCF from chicken induced activation of the PI3K/AKT and the MEK/ERK pathways in the chicken PGCs. In the published culture protocol the source of SCF is omitted but as it was added in quantified amounts it was assumed that the source must have been commercially available and therefore not chicken SCF. Although it has been documented that removal of additional SCF and FGF2 from the culture medium results in the dedifferentiation of the PGCs to EG cells (van de Lavoir *et al*. 2006) it is possible based on the results presented in this thesis that removal of SCF alone would not have affected the cultures.

IGF1 induced signaling pathway is essential for growth and development in vertebrates and is evolutionarily conserved (Molyneaux 2003). IGF1 can activate the PI3K/AKT and MEK/ERK pathways and is involved in PGC migration, specification and proliferation (Schlueter, Sang, *et al*. 2007; Schlueter, Peng, *et al*. 2007). IGF1 is not added as a purified factor in the van de Lavoir culture medium. However, analysis of protein from PGCs cultured in the presence of hIGF1 showed that phosphorylation of AKT but not ERK1/2 was induced. It was demonstrated that mouse SCF is not functional in chicken PGCs it is therefore likely that rat and bovine SCF are also not functional in chicken cells. Therefore, components of the medium

that induced AKT phosphorylation in the PGCs may be due to the presence of IGF1 as opposed to SCF.

LIF promotes survival and self-renewal of PGCs (reviewed by Katz *et al*. 2007) through activation of the MEK/ERK and JAK/STAT pathways. Addition of LIF to culture medium has been implicated in the differentiation of PGCs to EG cell in mouse, rabbit and chicken (Matsui *et al*. 1992; Park and Han 2000; Kakegawa *et al*. 2008). LIF is not added as a purified growth factor to the chicken PGC culture medium. However, LIF is a component of BRL conditioned medium (Smith *et al*. 1988) and the presence of a LIF-expressing feeder layer has been shown to inhibit mouse PGC differentiation (Chuma and Nakatsuji 2001; Farini *et al*. 2005). When LIF from chicken mouse and human were all added individually to starved PGCs cultures and the isolated protein sample probed, no phosphorylation of either ERK1/2 or STAT3 was detected. For the mouse LIF treated samples this was expected as Horiuchi *et al* (2004) showed that mouse LIF was not functional in chicken cell lines. As human LIF shares greater identity with mouse than chicken it is likely that the hLIF was also not functionally conserved with cLIF explaining the lack of phosphorylated proteins indicating a lack of pathway activation in the chicken PGCs. Interestingly no phosphorylated protein was detected in the samples treated with cLIF. This lack of induction in either pathway indicated that the cLIF was used at concentrations too low to be effective or was stored inappropriately, several months at 4^{o}C, resulting in a lower than expected cytokine activity. Chicken LIF is currently not commercially available and there is no antibody to carry out ELISA quantification. This means it is impossible to accurately quantify cLIF produced from transfected rat fibroblast cells. Without proper validation of cLIF it is not possible to draw conclusions from the absence of pathway activation.

Phosphorylated ERK1/2 was detected in PGCs cultured in the presence of chicken sera or FBS. Both chicken sera and FBS contain unknown levels of various growth factors, one or more of which must be activating the MEK/ERK pathway. Likely factors include FGFs, SCF and IGF1. Chicken sera did not induce activation of the

PI3K/AKT or JAK/STAT pathways. It is therefore unlikely that cSCF or cIGF1, known activators of these pathways, are inducing phosphorylation of ERK1/2. This indicated that FGF2 might be the activating factor. The FBS result supports this as it is unlikely that bovine SCF will be functionally active and if it was bovine IGF1 inducing the activation it might be expected that phosphorylated AKT would have been detected. From these observations it is highly likely that bovine FGF2 was present in the FBS and induced phosphorylation of ERK1/2. The lack of FGF2 mediated activation PI3K/AKT (Kimura *et al* 2007) is not unexpected and explained by the results from the protein isolated from the cells treated with purified hFGF2 where phosphorylated ERK1/2 but not AKT was detected.

BRL-conditioned medium contains several growth factors, LIF, IGF1 and SCF (Smith *et al*. 1988; Zsebo *et al*. 1990). BRL-conditioned medium induced AKT and ERK1/2 phosphorylation. Any SCF or LIF present in the BRL-conditioned medium will be from rat or bovine. SCF from mouse, rat and bovine are well conserved and as mouse SCF was not functional in chicken PGCs it was unlikely that bovine or rat SCF would be either. The same is true of LIF. These assumptions based on previous observations indicated that the observed induction of the PI3K/AKT pathway by BRL-conditioned medium might be attributed to presence of IGF1. This does not explain activation of the MEK/ERK pathway, which was not induced by recombinant hIGF1. These results indicated that the phosphorylation of ERK1/2 is mediated by another factor present in the BRL-conditioned medium. From the results presented here it is predicted that the ERK1/2 phosphorylation was mediated by FGF2 provided by the FBS that is a component of BRL-conditioned medium.

The results from the inhibitor experiments indicated that JAK/STAT signalling was required for chicken PGC propagation *in vitro*. Detection of phosphorylated STAT3 in protein samples from controls confirmed that the pathway was activated in PGCs cultured under normal culture conditions. However phosphorylated STAT3 was not detected in any of the protein samples from cells treated with individual components of the culture medium. These results indicated that no single component of the

culture medium was individually responsible for activation of the JAK/STAT pathway in the chicken PGCs. It is interesting that under normal conditions is STAT3 is phosphorylated yet no JAK/STAT activation was induced by individual components. This result and the requirement for JAK/STAT signalling shown in the inhibitor experiments may be explained by pathway interaction. As LIF activates the MEK/ERK pathway via JAK perhaps the effect on PGC proliferation observed using the JAK inhibitor was caused by a down regulation of this pathway and not the JAK/STAT pathway. Western blot analysis of cells treated with the inhibitor, for phosphorylated ERK and STAT3 would be able to determine which of the pathways was being inhibited.

Having established that several of the components of the van de Lavoir culture medium induce the same pathways it is possible to begin establishing a feeder- and serum-free culture system. The initial step would be the systematic removal of individual components of the medium and replacement with purified growth factors that induce the signalling pathways. The most appropriate place to start would be the removal of the BRL-conditioned medium. The BRL-conditioned medium is probably the most variable component of the culture medium. This medium is conditioned on a confluent layer of BRL cells for three days. Changes in the amount of growth factors and cytokines including LIF, IGF1 and SCF secreted by the cells during the conditioning of the medium is likely to vary between batches depending on the health of the cells, their growth rate and differences in confluence of the cells over the course of producing the BRL-conditioned medium. BRL conditioned medium also contains added FBS that will also have unknown amounts of growth factors, cytokines and hormones. BRL-conditioned medium induced the MEK/ERK and PI3K/AKT pathways. It might therefore be possible to remove the requirement for BRL-conditioned medium by adding hIGF1 and increasing the amount of added hFGF2 to compensate for a decrease in growth factors required to induce essential signalling pathways. Adding increased amounts of these growth factors may also be sufficient to remove the requirement of the STO-feeder cell line. Although BRLs are rat cells and STO from mouse it is likely that the factors secreted from each cell line

will be similar. Therefore activation of signalling pathways induced by STO-feeder cell secreted factors is likely to be the same as those in BRL-conditioned medium that also induce the pathways.

Alternatively the starting point towards a more robust culture system could be the removal of the requirement for growth on a layer of feeder cells. In the inhibitor experiments the survival and proliferation of the control PGCs cultured for seven days in the absence of a feeder layer was the same as in the presence of one. This suggests that the feeder cell layer may in fact not be required for PGC culture using the culture conditions described in this thesis. This is supported by the work of Choi *et al* (2010) where it was demonstrated that removal of the feeder layer could be counteracted by increasing the amount hFGF2 added to the culture medium. They also suggest that LIF and SCF added to the medium support the propagation of the chicken PGCs on removal of a feeder layer. Although not highlighted in the discussion of the results the data presented by Choi *et al* (2010) also indicates that only increased amounts of hFGF2 are actually necessary to support PGC growth. Based on the results of Choi *et al* (2010) and the results presented in this thesis it is unlikely that SCF or LIF produced from the feeder layer induce signalling pathways in the chicken PGC. It was shown here that SCF from chicken but not mouse could induce PI3K/AKT and MEK/ERK activation. Although LIF induction wasn't observed and as mentioned previously published data shows that LIF from human is not functional in chicken cells (Horiuchi *et al*. 2004). Mouse LIF was shown to be more related to human, based on amino acid sequence alignment so it was assumed that it would not be active in chicken cells.

The results presented in this thesis indicate that both MEK/ERK and PI3K/AKT signalling are essential for chicken PGC propagation in culture. It has been shown that these pathways are stimulated by more than one component of the culture medium suggesting that the medium could be simplified by systematic replacement of components containing undetermined factors with purified growth factors. It has been shown that growth of the cells on a feeder layer may not be required and given

the results for Choi *et al* (2010) may be simply replace by increasing the amount of hFGF2 added to the culture medium. The data indicated that whilst SCF and LIF from mouse, rat and bovine were not functional and did not activate signalling pathways in the chicken PGCs that IGF1 from these species did.

6.1.3 Transposon-derived vectors offer significant potential in the development of transgenic chickens

Until now genetic modification of chicken PGCs cultured long term *in vitro* has been relatively unsuccessful with stable transfection rates of no more than 0.001%. Stable transfection of chicken PGCs using plasmid DNA was not achieved until the insertion of insulator sequences flanking the reporter gene were used as a barrier to gene silencing as a result of epigenetic rearrangements. Incorporation of insulator sequences flanking the transgene improved the stable transfection efficiency only minimally from 0 to $5x10^{-7}$% (van de Lavoir *et al*. 2006). Using the ΦC31 integrase system Leighton *et al*. (2006) showed that stable transfection of chicken PGCs could be significantly improved, demonstrating more than a 10,000 fold improvement in efficiency compared to what was reported by van de Lavoir *et al* (2006) both with and without the incorporation of insulator sequences. However, these efficiencies were still very poor, 0.001%. In this thesis data has been presented on the use of *Tol2* and piggyBac transposon-derived vectors for the trangenesis of chicken PGCs. Stable transfection of the PGCs was determined by expression of GFP three weeks post transfection of the cells. It was shown that even in the absence of insulator sequences these transposon-derived vectors can be used efficiently to stably transfect transgenes into chicken PGCs. It was calculated that these vectors were 50 to 370 fold more efficient at producing stably transfected PGCs than the ΦC31 integrase system used by Leighton *et al*. (2006). Interestingly expression of transgenes that were integrated using the transposon-vector system did not seem to be effected by gene silencing. It was shown that in both the DF1 and PGCs transfections no significant increase in stable transfection rate was observed between cultures transfected with the piggyBac vector with insulator sequences and those transfected with the piggyBac vector without insulator sequences. Notably significantly more

PGCs were stably transfected using the piggyBac vector without insulators (PB-CGIP) than the vector with (PB-CGIP-HS4). Transposon size can result in a decrease in transposition efficiency (Izsvak *et al*. 2006) but for piggyBac-derived vectors transposition efficiency only decreased when inserted DNA exceeded 9kb. The size of the inserted DNA in vector PB-CGIP-HS4 was approximately 5kb. Despite this it is thought that this increase in vector size may be resulting in the observed reduction in efficiency. Overall the stable transfection efficiencies observed here, 5% for PB-CGIP-HS4, 10.5% for PB-CGIP and 37% for pTol2-CGIP far exceed those achieved using any other method for the trangenesis of chicken PGCs to date.

Chicken PGCs stably expressing a myrGFP reporter construct inserted by piggyBac-mediated transposition were shown to retain the ability to form functional sperm. Cells were injected into embryos and a male germ line chimera was produced. This cockerel was tested and shown to transmit the donor PGCs at an efficiency of 0.4%. Low transmission rate was predicted to result from toxicity of the membrane localised expression of the GFP marker gene. This conclusion was based on observations within the laboratory that were discussed in chapter 5.

The research presented here makes significant advances in the modification of PGCs. It showed that both piggyBac and *Tol2*-derived vectors are extremely efficient systems for the production of transgenic PGCs. In particular the *Tol2*-derived vector was shown to be the most effective method for the transgenesis of cultured chicken PGCs to date. Overall it has been clearly demonstrated that transposon-derived vector systems are a useful tool for the efficient production of transgenic PGCs.

6.2 CONCLUDING REMARKS

The *in vitro* culture of chicken PGCs offers significant potential for germ cell research and the development of transgenic birds. Since the publication of the van de Lavoir *et al*. (2006) method for the culture of PGCs, little research has been published utilising chicken PGCs. This may be due to difficulty in recapitulation of the van de Lavoir *et al*. (2006) method that was highlighted in this thesis. Data presented here has shown that by modifying the method, it is possible to establish PGC lines that can be propagated in culture long term. Transposon-derived vectors were shown to be much more efficient at genetically modifying chicken PGCs than any previously published method and the modified cells retained the ability to form functional gametes. The PGC cultures that were established during this project have been used to gain a better understanding of primordial germ cell biology and may give insight it to the development of a feeder- and serum- free culture system. Development of such a system would provide a more robust method for the establishment of PGC cultures. Without a more robust culture method the potential of chicken PGCs in research or industrial applications may go unrealised.

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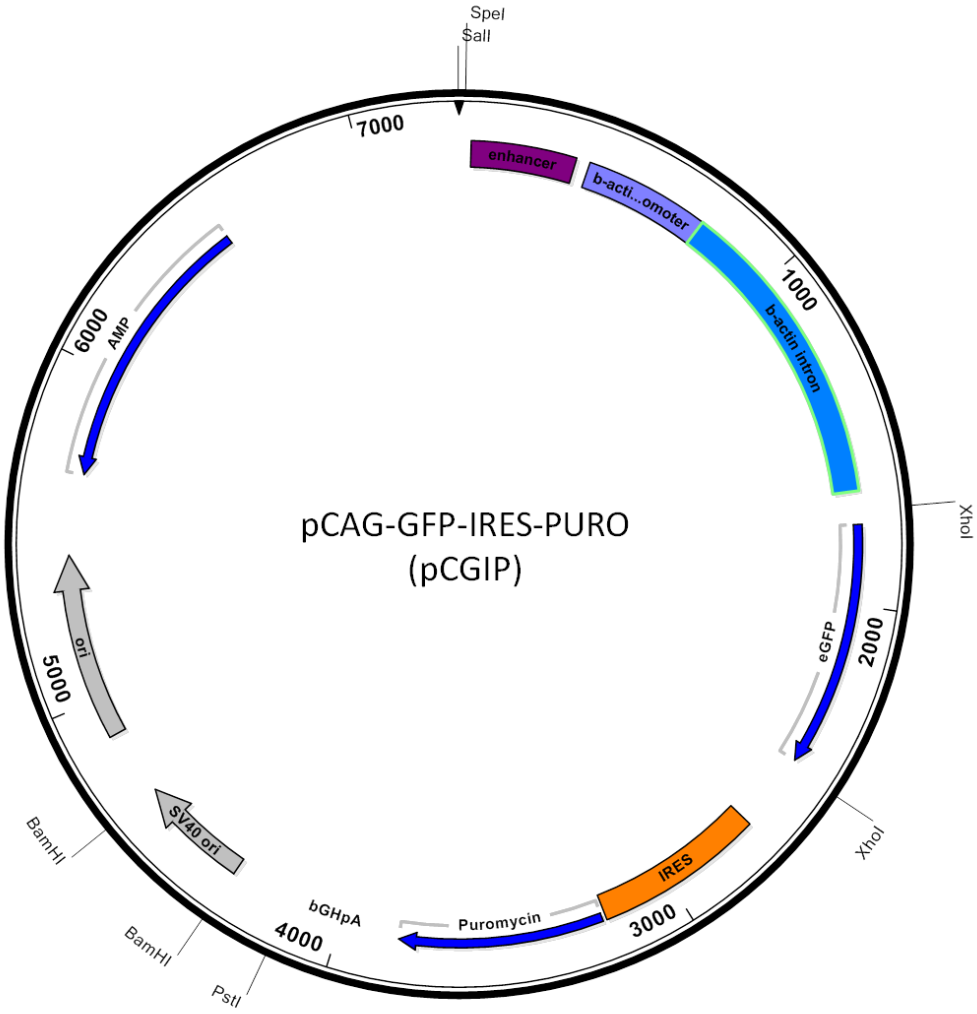
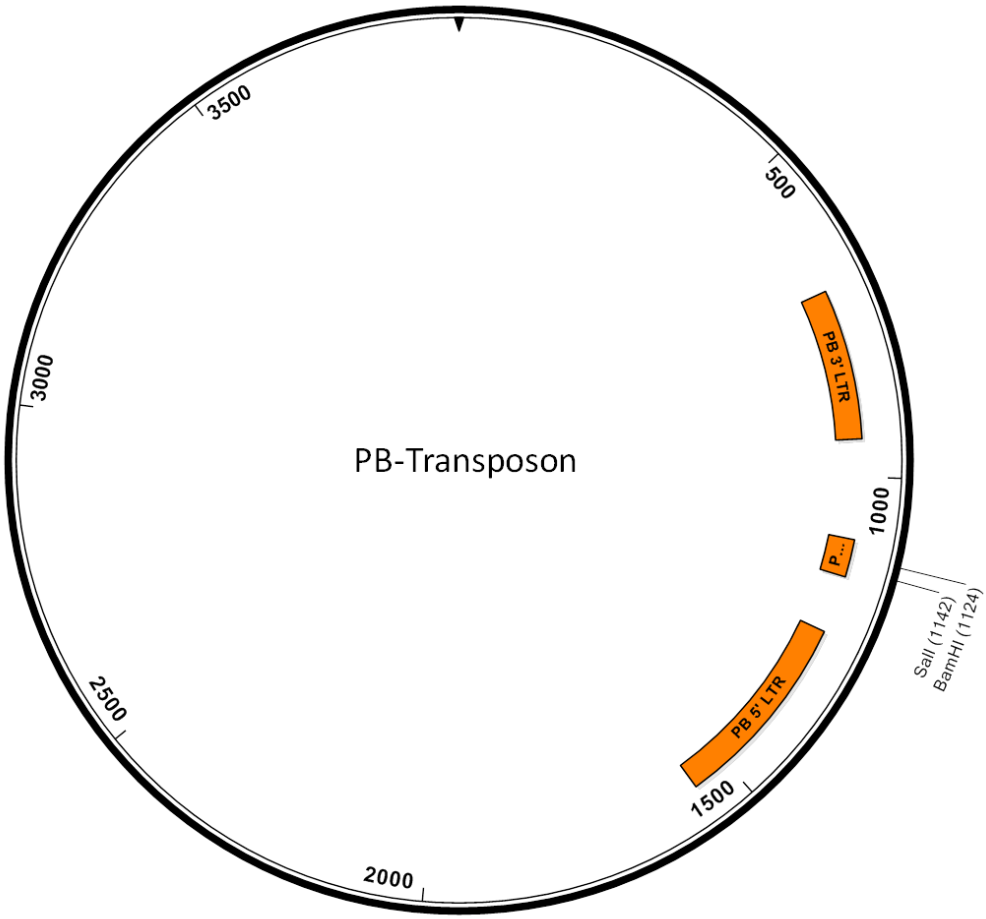
APPENDIX A: VECTOR MAPS

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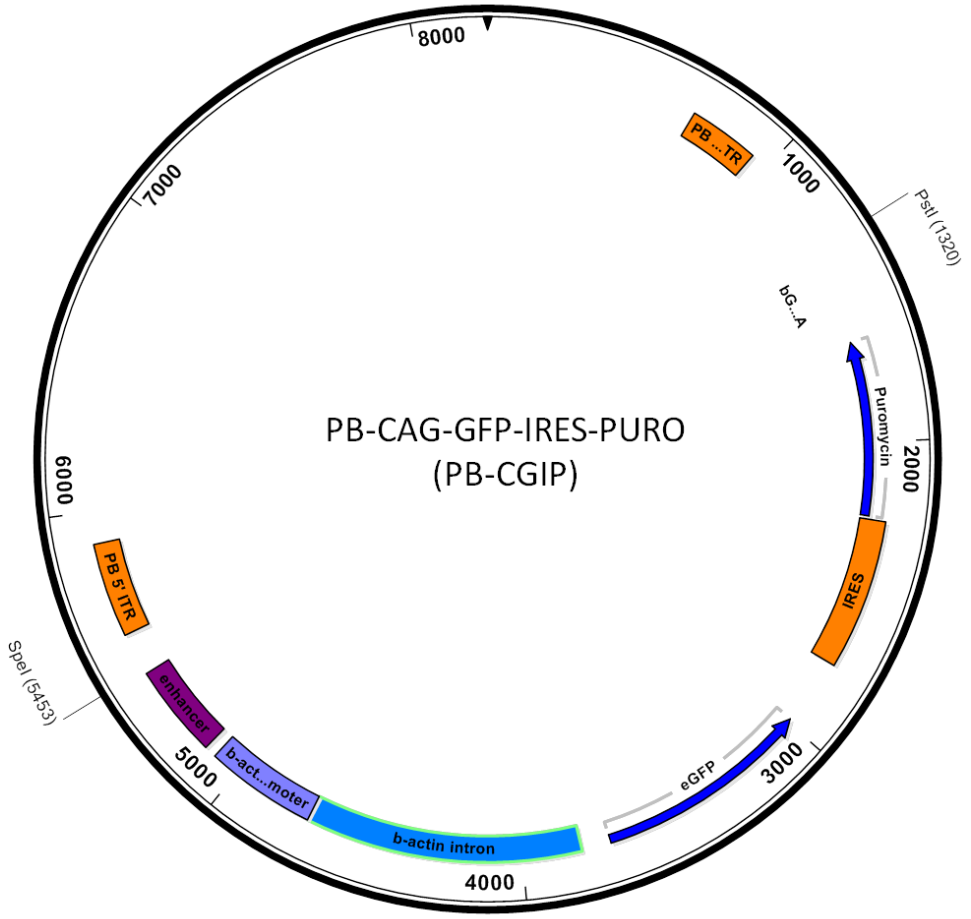
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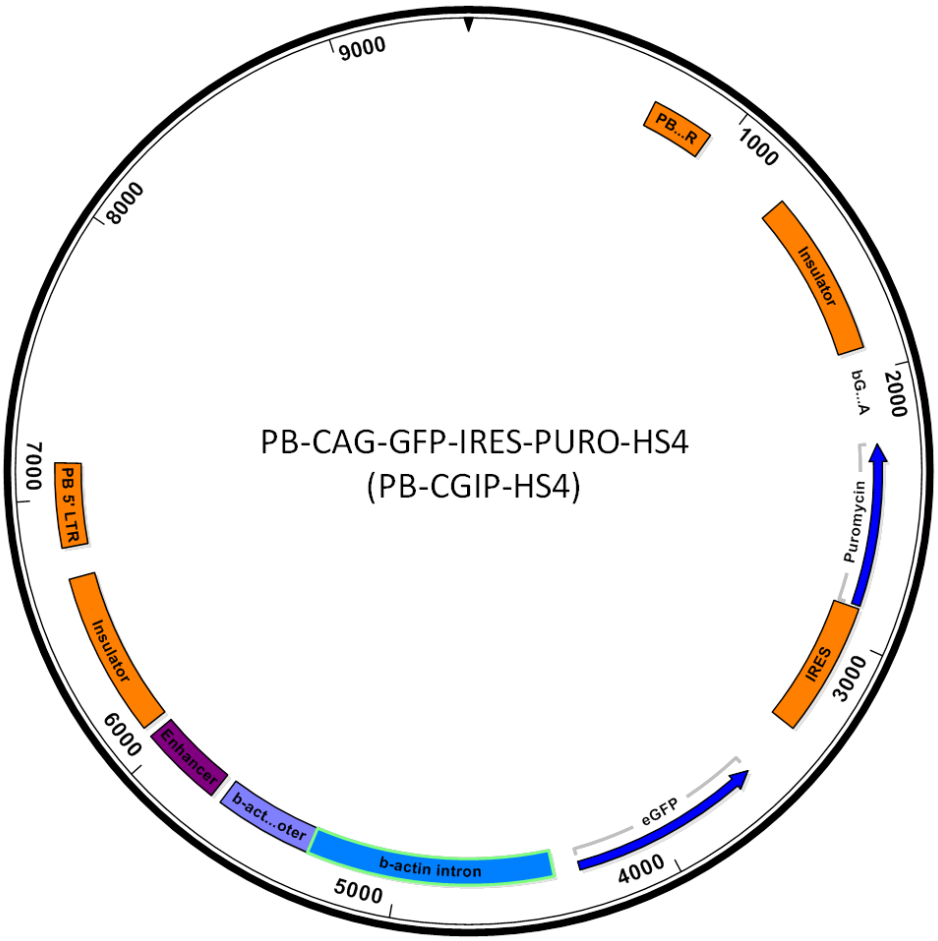
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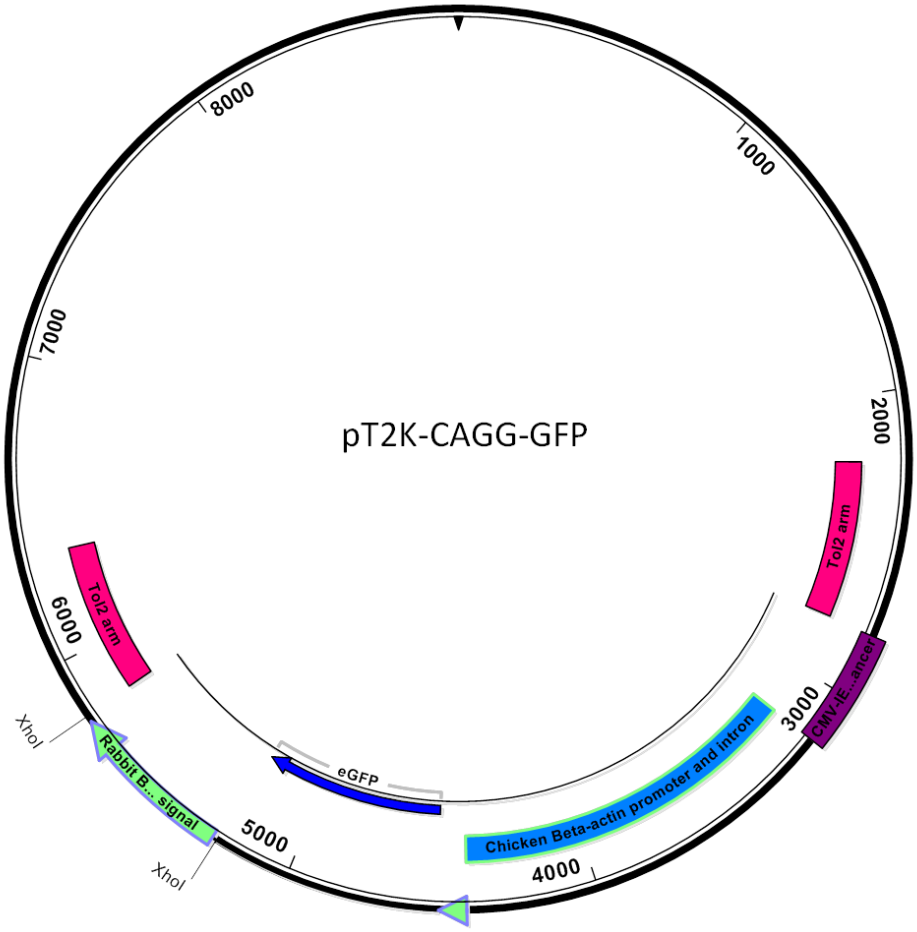
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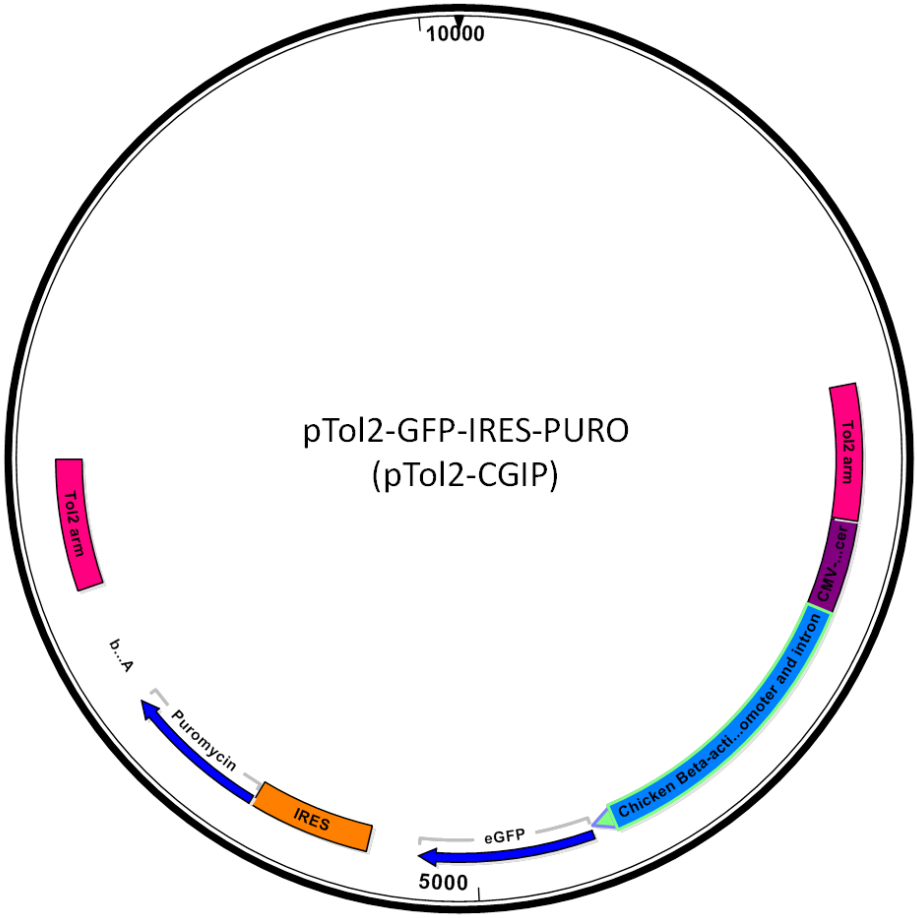
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Puromycin
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APPENDIX B: RELEVANT PUBLICATIONS

OPEN ACCESS Freely available online

PLoS one

Characterisation and Germline Transmission of Cultured Avian Primordial Germ Cells

Joni Macdonald, James D. Glover, Lorna Taylor, Helen M. Sang, Michael J. McGrew*

The Roslin Institute and Royal Dick School of Veterinary Studies, University of Edinburgh, Roslin, United Kingdom

Abstract

Background: Avian primordial germ cells (PGCs) have significant potential to be used as a cell-based system for the study and preservation of avian germplasm, and the genetic modification of the avian genome. It was previously reported that PGCs from chicken embryos can be propagated in culture and contribute to the germ cell lineage of host birds.

Principal Findings: We confirm these results by demonstrating that PGCs from a different layer breed of chickens can be propagated for extended periods *in vitro*. We demonstrate that intracellular signalling through PI3K and MEK is necessary for PGC growth. We carried out an initial characterisation of these cells. We find that cultured PGCs contain large lipid vacuoles, are glycogen rich, and express the stem cell marker, SSEA-1. These cells also express the germ cell-specific proteins CVH and CDH. Unexpectedly, using RT-PCR we show that cultured PGCs express the pluripotency genes *c-Myc, cKlf4, cPouV*, *cSox2*, and *cNanog*. Finally, we demonstrate that the cultured PGCs will migrate to and colonise the forming gonad of host embryos. Male PGCs will colonise the female gonad and enter meiosis, but are lost from the gonad during sexual development. In male hosts, cultured PGCs form functional gametes as demonstrated by the generation of viable offspring.

Conclusions: The establishment of *in vitro* cultures of germline competent avian PGCs offers a unique system for the study of early germ cell differentiation and also a comparative system for mammalian germ cell development. Primary PGC lines will form the basis of an alternative technique for the preservation of avian germplasm and will be a valuable tool for transgenic technology, with both research and industrial applications.

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Competing Interests: The authors have declared that no competing interests exist.

* E-mail: mike.mcgrew@roslin.ed.ac.uk

Introduction

Primordial germ cells (PGCs) are the precursors of the germ cell lineage and are restricted to the formation of sperm and eggs in the adult organism. In mammals, PGCs are specified at the beginning of gastrulation. In contrast, in avian species the germ cell lineage is segregated from somatic cell lineages in the epiblast of the laid egg [1]. Early germ cell precursors in chicken embryos can be identified by the expression of the germ cell-specific protein, chicken vasa homologue (CVH) [2]. From a position in the central epiblast, PGCs migrate to an extraembryonic region anterior to the future head region, termed the germinal crescent. From here, at three days of development (stage 15 HH, [3]), the PGCs invade the forming vascular system, congregate in the lateral plate mesoderm conjoining the future gonadal region, and actively populate the developing gonads over the subsequent 48 hours [4]. In the gonad, these primitive germ cells differentiate in accordance with the sexual identity of the surrounding tissues. In the female, germ cells enter meiosis at day 16 of incubation whereas in the male germ cells undergo mitotic arrest and give rise to spermatogonial stem cells which produce functional spermatozoa, beginning at approximately 16 weeks post-hatch.

PGCs in mouse are specified from a region of caudal extra-embryonic mesoderm, much later during embryonic development than in the chicken and can only be propagated for short periods in culture [5]. In specific cell culture conditions, mouse PGCs will 'de-differentiate' into cells resembling ES cells, termed EG (embryonic germ) cells [6,7]. This change in cell fate is thought to occur as mouse PGCs already express several pluripotency markers and respond to growth factors present in the culture medium [8]. A similar de-differentiation process may occur during the formation of germ cell teratomas during embryogenesis [9]. Chicken PGCs can also form EG cells in culture, but it is not known which pluripotency genes are expressed by these cells during this process [10,11,12].

It was reported that migratory PGCs could be isolated from the blood of Barred Plymouth Rock layer chickens and expanded in culture for several months [12]. When transplanted to same-sex recipient embryos at stage 13–15 HH, these cells differentiated into functional gametes and generated viable offspring whose genotype derived from the cultured PGCs. Transplantation of the cultured PGCs into opposite-sex recipient embryos did not result in donor-derived functional gametes and the developmental fate of the PGCs in these embryos was not determined.

A robust culture system for chicken PGCs could form the basis of an *in vitro* system for the study of genetic pathways involved in early germ cell proliferation and survival. This will advance our understanding of the mechanisms of early germ cell development

and also provide a comparative system which will be informative for studies on mammalian germ cell development. Germline competent PGCs can be developed as a cell-based genetic modification system for the chicken, providing a valuable tool for transgenic technology with both research and industrial applications [13,14]. This is required as isolated lines of chicken ES (cES) cells do not contribute to the germline after short periods in culture [15,16,17]. The only process available for germplasm preservation in poultry is the cryopreservation of semen, which in itself is variable in terms of recovery of functional semen for artificial insemination [18,19]. Since it is not possible to cryopreserve chicken oocytes and embryos, the development of PGC culture and cryopreservation protocols will provide a means to preserve the germplasm of both males and females and recover the full genetic complement of an avian breed or species.

The key question addressed in this study was whether migratory PGCs could be isolated and cultured from a further breed of chickens and form functional gametes and viable offspring. In addition, we also investigated the intracellular signalling pathways necessary for PGC growth and the pluripotency genes and germ cell-specific markers expressed by cultured PGCs.

Materials and Methods

PGC culture conditions

2µl–4µl of blood was isolated from the vasculature system of stage 15–16 HH stage embryos of ISA Brown hens inseminated by ISA brown roosters. Blood was also collected from ISA Brown embryos carrying a single copy lentiviral integrant that contains a transgene that expresses green fluorescent protein (GFP) ubiquitously (Roslin Greens, [20]). Embryos were sexed using primers specific for the W chromosome as described in [21]. Each blood sample was split between two wells of a 48 well tissue culture dish containing 3.0×10^4 irradiated STO (Sandoz inbred mouse-derived thioguanine-resistant and ouabain-resistant) feeder cells per well and 0.3 ml of PGC culture medium with or without additional growth factors. One third of the culture medium was changed every two days until PGC outgrowth was observed. Thereafter, the total volume of medium was changed every two days. PGC culture medium used was essentially as described in [12] with some modifications. Medium contained 50% BRL (buffalo rat liver) conditioned medium in KO-DMEM (Invitrogen) and contained 10% Fetal Bovine Serum (FBS) (ES cell tested, PAA Laboratories), 2.5% chicken serum (Biosera or Sigma), 2 mM GlutaMax (Invitrogen), 1× NEAA (Invitrogen), 0.1 mM â-mercaptoethanol (Invitrogen), 1× nucleosides (Invitrogen), 1 mM pyruvate (Invitrogen), 1× Penicillin-Streptomycin (Sigma). Growth factors (human bFGF, mouse and human SCF) were obtained from R&D Biosystems. Charaterised FBS (Hyclone) and PAA-Gold FBS (PAA Laboratories) did not support PGC derivation under these conditions, n = 1/151 and n = 0/60, respectively. Inhibitors were obtained from Calbiochem (LY294002 and PD0325901) and prepared according to manufacturer's protocols. Cells were treated with inhibitors (LY294002, 10 µM [22]) (PD0325901, 1 µM [23]) or vehicle every two days.

Immunohistochemistry and *in situ* hybridisation analysis

PGCs were fixed using 4% paraformaldehyde in PBS for 10 min at room temperature. Primary antibodies were added (rabbit anti-CVH (1:250), rabbit anti-CDH (1:250), mouse anti-Tuj III (1:200, Covance), mouse anti-SSEA1 (1:40, Developmental Studies Hybridoma Bank)), in 5% goat serum/PBT and samples were incubated overnight at 4°C. Cells were washed for 30 min in PBT and re-incubated with secondary antibodies for one hour (goat anti-rabbit IgG Alexa-Fluor 488, donkey anti-mouse IgG Alexa-Fluor 543, or rabbit anti-mouse IgM Alexa-Fluor 546 for the SSEA1 antibody). Cells were washed for 30 min, counter-stained with Hoechst (Sigma), mounted in PBS and imaged directly. The cellular fluorescent stains HCS LipidTOX Green and Mito tracker Red FM CMXRos were used following manufacturer's protocols (Invitrogen). Cells were imaged using an inverted confocal microscope (Nikon eC1; Nikon Instruments). Images were captured using Nikon EZ-C1 Software v3.40.

Whole mount in situ hybridisations were carried out as described [24]. The riboprobe to *cPouV* was described in [25].

RNA isolation and cDNA synthesis

Total RNA was isolated from cells using RNeasy minikit (Qiagen) according to the manufacturer's guidelines. For cDNA synthesis 1 µg of RNA was heat-treated at 70°C for 10 min and added to the following 20 µl reaction mix: 25 mM $MgCl_2$, 4 µl; 10× reverse transcription buffer, 2 µl; 10 mM dNTP mixture, 2 µl; recombinant RNasin, 0.75 µl; random primers, 0.5 µl. Samples were incubated at room temperature for 10 min; 42°C for 55 min; 95°C for five min using the Reverse Transcription System (Promega). For negative controls, the reactions were carried out without reverse transcriptase.

Reverse Transcription PCR

A 15 µl reaction mixture containing 6 µl H_2O, 1.5 µl 10× buffer (Roche), 0.3 µl 10 mM dNTPs (Invitrogen), 0.3 µl each primer (50 pmol/µl), 0.1 µl Fast Start Taq (Roche), 3 µl 5× creosol red, and 2 µl sample cDNA. The following reactions were carried out: 95°C for 20 min, followed by 30 cycles of 95°C for 30 sec, annealing temperature for 30 sec, 72°C for one min, and a final extension of 60°C for 30 min. Samples were resolved on a 0.9% TAE agarose gel. Primer sets and annealing temperatures were:

cPouV: TCAATGAGGCAGAGAACACG, TCACACATTTGCGGAAGAAG 58°C

cvh: AGCACAGGTGGTGAACGAACCA, TCCAGGCCTCTTGATGCTACCGA 58°C

c-Myc: GCACAGAGTCCAGCACAGAA, GTTCGCCTCTTGTCGTTCTC 50°C

cKlf4: AGCTCTCATCTCAAGGCACA, GGAAAGATCCACTGCTTCCA 50°C

cSox2: AGGCTATGGGATGATGCAAG, GTAGGTAGGCGATCCGTTCA 50°C cGapdh: CAGATCAGTTTCTATCAGC, TGTGACTTCAATGGTGACA 58°C

cNanog: TTGGAAAAGGTGGAACAAGC, GGTGCTCTGGAAGCTGTAGG 60°C

Y-irradiation

Fertile eggs (ISA Brown) were irradiated at the laid egg stage prior to incubation using a MDS Nordion Gammacell 1000 Elite with a Cs^{137} source.

PGC transplantation and host embryo culture

Germline chimeras were generated by injection of GFP^+ PGCs into the cardiac tract of stage 16 HH embryos. Embryos were transferred into phase III host shells and cultured to hatching as described [26]. The hatched chicks were raised to sexual maturity and genomic DNA samples extracted from semen of adult roosters were screened by semi-quantitative PCR to identify roosters carrying the GFP transgene in the germ cell lineage [27]. Briefly, PCR was carried out on 50 ng of genomic DNA using primers specific for the transgene (CGAGATCCTACAGTTGGCGCCC-

GAACAG; ACCAGTAGTTAATTTCTGAGACCCTTGTA, annealing temperature: 58°C). In order to estimate the copy number, control PCR reactions were carried out in parallel using 50 ng of non-transgenic DNA spiked with vector plasmid DNA in varying amounts to give the equivalent concentration of one copy per genome (100%), one copy per 10 genomes (10%), one copy per 100 genomes (1%), or one copy per 1000 genomes (0.1%). Founder roosters identified by this method were crossed to stock hens. Offspring were screened for GFP fluorescence to identify birds deriving from the PGCs. All experiments described in this report involving animals, animal breeding, and animal care procedures were reviewed and approved by The Roslin Institute's animal ethics committee. These experiments were performed under specific license from the U.K. Home Office.

Culture of chicken ES cells

Chicken embryonic stem (cES) cells were isolated and cultured as described in [28] with some changes. The epiblast of GFP^+ laid eggs was isolated, dissociated, and cultured on either STO or BRL feeder cells in PGC medium containing 80% BRL conditioned medium and 5 ng/ml bFGF. Chicken ES cells were expanded for four to six weeks before mRNA was isolated from two independent lines as described above for the PCR analysis. The two lines of cES cells were further tested for pluripotency after an additional four to six weeks in culture by injection into the sub-germinal cavity of newly laid eggs that were first irradiated at 5.0 Gray (Gy). cES were dissociated from a 24 well plate using cell dissociation buffer (Invitrogen). Cells were resuspended in KO-DMEM and 1 ul of solution (~500–1000 cells) was injected into the sub-germinal cavity. Injected eggs were transferred to phase II host shells [26] and incubated in these shells for eight days without transfer to new host shells. An embryo containing GFP^+ cells from each line was cryosectioned to assay for GFP^+ cell contribution to host tissues.

Statistical analysis of inhibitor experimental data

For PGC culture derivation the no added growth factors condition was compared individually to each of the other culture conditions and the data statistically validated using a Paired Student T-Test with two tailed distribution. A Paired Student T-Test with two tailed distribution was also used to compare the data from the inhibitor experiments where vehicle was compared with experimental.

Results

Propagation of PGCs *in vitro*

Long term *in vitro* culture of PGCs and germline transmission has been demonstrated for PGCs deriving from Barred Plymouth Rock chickens [12]. We attempted to repeat and extend this investigation using a different breed of layer-type chickens, the ISA Brown. Embryonic blood containing migratory PGCs was isolated from day 3 (Stage 16 HH) embryos and cultured on a layer of STO feeder cells. Culture medium contained both chicken and fetal bovine animal sera, conditioned medium from BRL cells, bFGF and SCF (see Materials and Methods). After two weeks in culture PGCs were present in the majority of culture wells and by three weeks blood cells in the wells had lysed. The cells remaining in several wells per experiment displayed the described morphology of PGCs (Fig. 1) [12,29].

We carried out a large number of experiments in parallel, to determine which commercially-available FBS and chicken sera supported PGC survival and which growth factors were required as additives to the basic medium. We defined a successful culture derivation as more than 100 PGCs being present in the culture at the end of three weeks. Several sources of fetal bovine and chicken sera did not support the growth of PGCs (see Materials and Methods). Using selected serum conditions (Materials and Methods) we assayed if the addition of bFGF and SCF improved the frequency of PGC culture derivation (n = 370) (Fig. 1). We found that addition of bFGF significantly increased PGC culture derivation but addition of SCF did not. Several lines of cultured PGCs were expanded from single embryo blood samples (seven lines, cell number >100,000 for each) and used for the subsequent experiments. PCR analysis of these lines for a female-specific W chromosome [21] revealed that all lines isolated were male.

Propagation of PGCs is dependent on PI3K and MEK signalling

We assayed the effect of inhibiting phosphatidylinositol-3kinase (PI3K) on PGC propagation using the inhibitor, LY294002. PI3K is activated by many signalling pathways, including the c-kit receptor [30]. The c-kit ligand, SCF, is a known survival factor/mitogen for mouse primordial germ cells [31,32,33]. PGCs were grown in PGC culture medium containing inhibitor dissolved in vehicle or vehicle alone and cell number was assayed after one week. We observed that PGC proliferation was severely inhibited in the presence of LY294002 (Figure 2A). Cells were assayed for viability by the cellular exclusion of trypan blue. Most cells in the inhibitor treated wells were trypan blue positive (90% inhibitor treated, <10% vehicle treated cells) after seven days indicating that cell death was increased in the presence of inhibitor.

We next assayed if the FGF/MAP kinase pathway was necessary for PGC proliferation by treating cultured PGCs with PD0325901, a potent inhibitor of MEK [34]. FGF has been shown to be a survival factor and activate MAP kinase in mouse migratory PGCs [35]. PGCs were again grown in medium containing inhibitor dissolved in vehicle or vehicle alone and cell number was assayed after one week. PGC number was significantly reduced in the presence of the MEK inhibitor (Fig. 2B). A trypan blue cellular exclusion assay revealed that cell death increased in the presence of the inhibitor (90% inhibitor treated, <10% vehicle treated cells) after seven days in culture. These results demonstrate that signalling through PI3K and MEK are necessary for PGC growth in culture.

Characterisation of cultured PGCs

To examine the cellular morphology of cultured PGCs and the intracellular localisation of germ cell-specific proteins, we carried out immunofluorescence on two PGC lines maintained *in vitro* for three months and 12 months. The cultured PGCs contain a large nucleus and many prominent vacuoles (Fig. 3). To determine the contents of the vacuoles we stained the PGCs with LipoTox, a marker of neutral lipids. This revealed that many of the larger vacuoles contain neutral lipid (Fig. 3A). We also carried out the classic Periodic acid-Schiff (PAS) reaction on the PGCs, a stain for cellular glycogen. PAS staining produced a diffuse staining pattern throughout the cytoplasm indicating a cytoplasm rich in glycogen particles (Fig. 3F). Staining with Mitotracker Red, an active mitochondrial marker also revealed dispersed functional mitochondrial throughout the cytoplasm (Fig. 3E). Immunostaining with the ES cell marker, SSEA-1 demonstrated that the cell surface of PGCs stained strongly for this epitope (Fig. 3D).

To determine if the cultured PGCs continued to express the germ cell-specific proteins found in migratory PGCs *in ovo*, we used immunofluorescence to detect CVH, chicken vasa homologue, and CDH, chicken dead end homologue; two RNA processing proteins important for germ cell survival and specification [2,36,37,38,39]. Immunostaining with an antibody to CVH

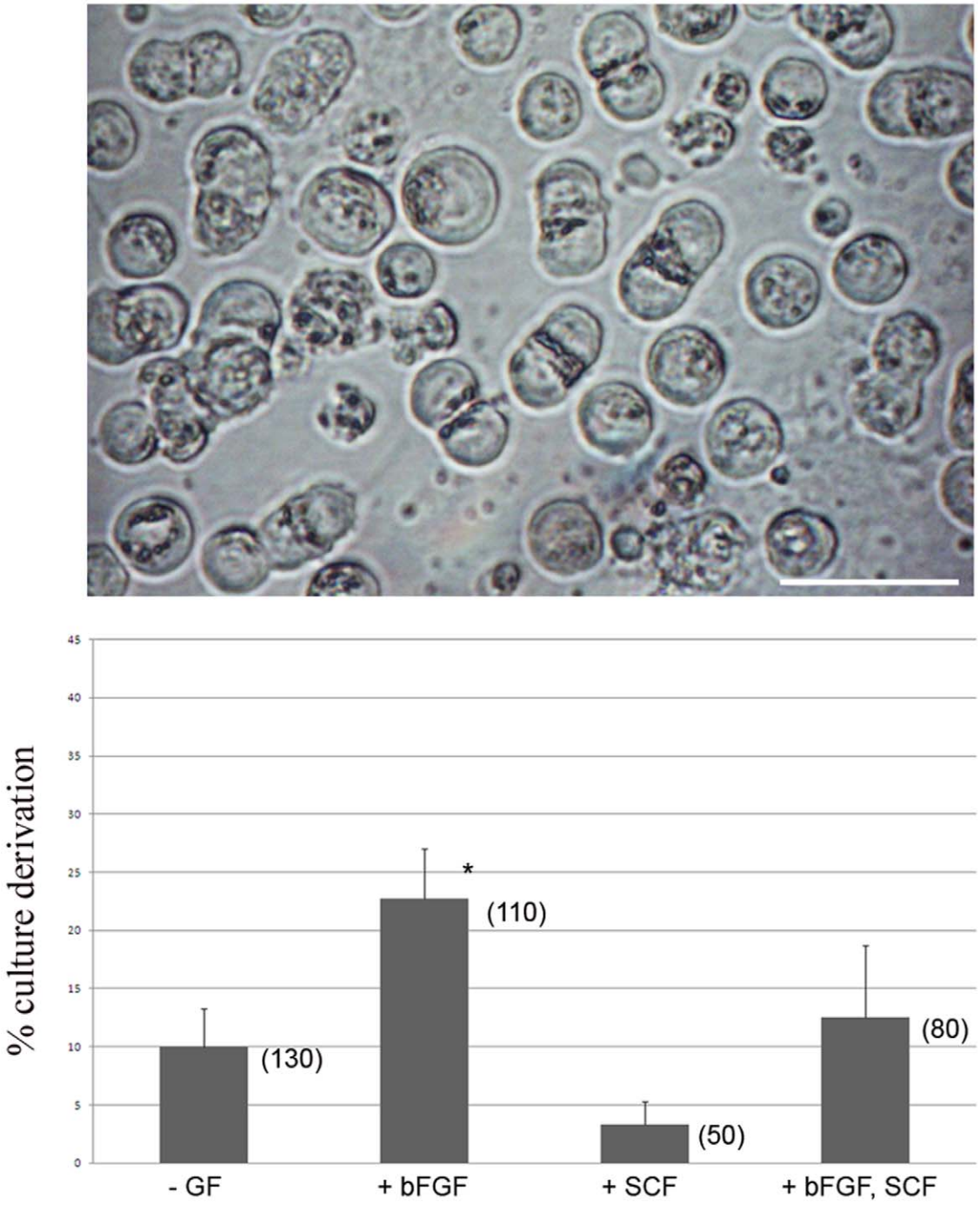

Figure 1. *In vitro* culture of PGCs. Top: PGCs from a representative culture were imaged using brightfield microscopy. Doublets indicative of dividing cells are visible in the culture. Bar, 50 μm. **Bottom:** Blood from single embryos was split into two wells and cultured with or without additional growth factors. Wells containing more than 100 non-adherent PGCs at three weeks were scored as positive. Cultures contained no additional growth factors or 2.5 ng/ml bFGF with or without 5 ng/ml SCF. (n) indicates the number of cultures assayed. Error bars, S.E.M. *, $p<0.05$.
doi:10.1371/journal.pone.0015518.g001

illustrated that in most cells CVH was localised throughout the cytoplasm (Fig. 3B). This is consistent with the reported cytoplasmic localisation of CVH in avian germ cells [2]. Immunostaining with CDH antibody displayed a strong nuclear localisation and diffuse staining throughout the cytoplasm (Fig. 3C). This result is consistent with the reported description of CDH as a nuclear-localised protein in migratory and post-migratory PGCs [39]. We conclude from these results that the expression of these germ cell-specific proteins is maintained in cultured PGCs.

Cultured PGCs express a set of pluripotency genes

We subsequently examined the expression of the known pluripotency markers *cPouV*, *cSox2*, *cNanog*, *cKlf-4*, and *c-Myc* in cultured PGCs. During germ cell specification in the mouse, nascent germ cells begin to express *Oct3/4*, *Nanog*, and *Sox2*, and

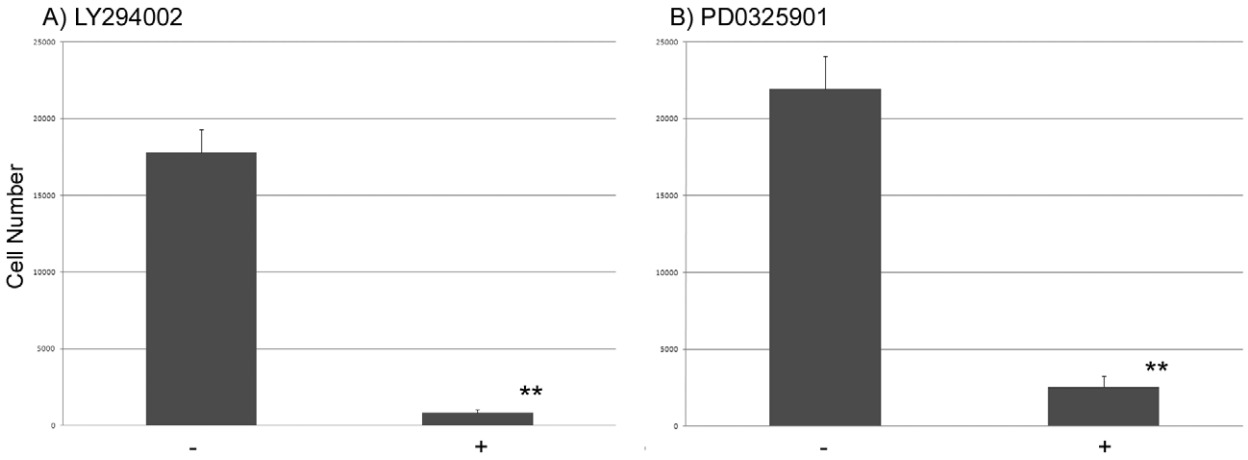

Figure 2. PI3K and MEK are necessary for PGC proliferation. PGCs (1000) were seeded into a well and grown in the presence of pharmalogical inhibitors or vehicle for seven days in medium containing 2.5 ng/ml bFGF and total cell number was assayed. **A**) LY294002, (10 μM). **B**) PD0325901, (1 μM). Three lines of cPGCs were assayed between 3–6 times in three separate experiments. Error bars, S.E.M. **, $p<0.01$.
doi:10.1371/journal.pone.0015518.g002

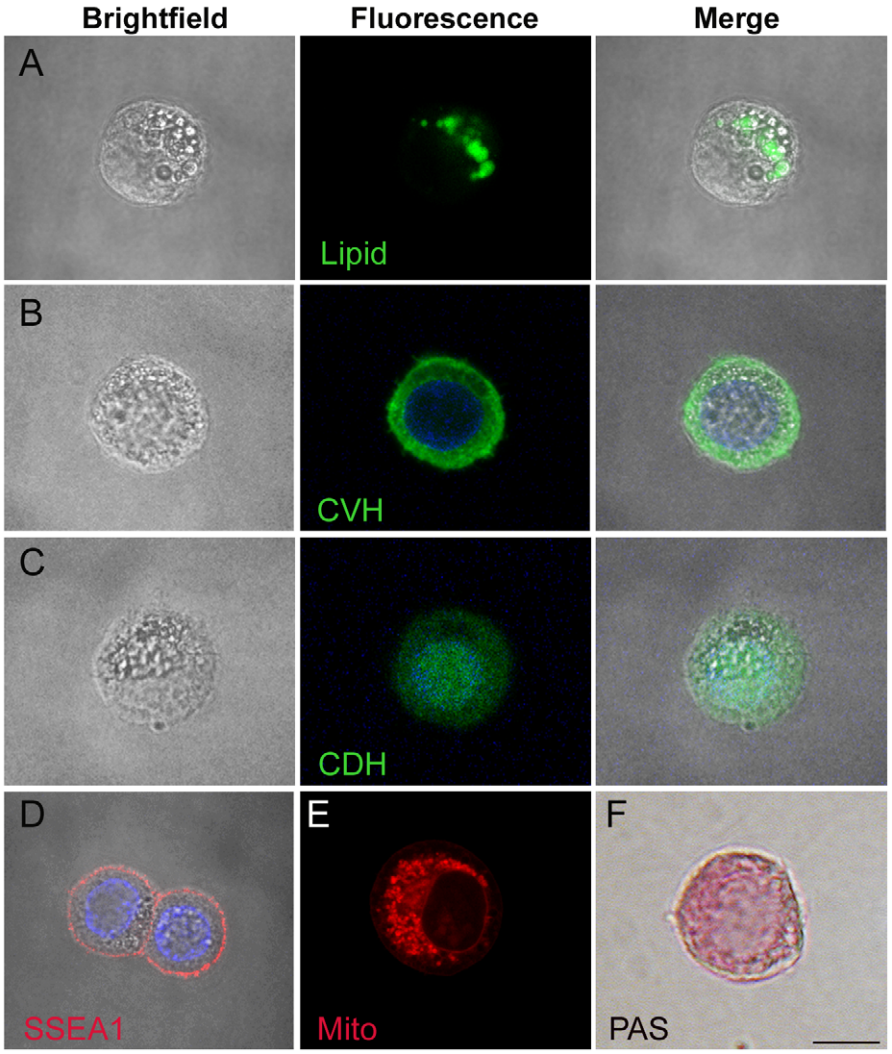

Figure 3. Sub-cellular localisation of germ cell markers in PGCs. Immunofluorescence of select germ cell markers was carried out on two separate lines of PGCs. Staining patterns for both lines were equivalent. **A**) LipoTox, a marker of neutral lipid. **B**) CVH, chicken vasa homologue. **C**) CDH, chicken dead end homologue. **D**) SSEA-1. **E**) Mito Tracker Red. **F**) PAS staining. Bar, 10μm.
doi:10.1371/journal.pone.0015518.g003

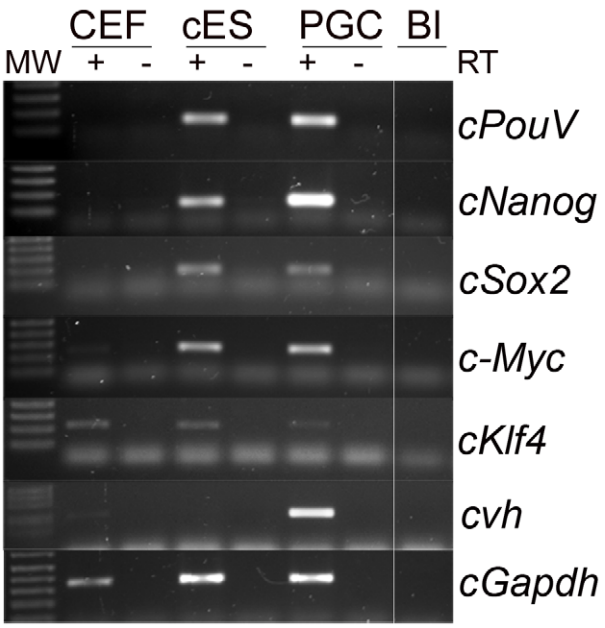

Figure 4. PGCs express many pluripotency genes. RT-PCR was carried out on cDNA samples from two independent lines of PGCs and cES cells. CEF, chicken embryonic fibroblasts; BI, blank-no cDNA control.
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express *c-Myc* and *Klf4* only upon conversion to embryonic germ (EG) cells [40,41]. We examined the expression of the chicken homologues of these four genes in cultured PGCs, cES cells, and chicken embryonic fibroblasts (CEFs). The cES cells used in this study were shown to contribute to the three germ layers of the forming chicken embryo in chimeras (Fig. S1). We isolated RNA from CEFs, STO feeder cells, cES cells and cultured PGCs and carried out RT-PCR analysis (Fig. 4). The germ cell-specific marker *cvh* was used as a positive control for PGC-specific gene expression and was found to be expressed in cultured PGCs and not in cES cells. We found that cES cells expressed all four of the pluripotency markers, *cPouV*, *cSox2*, *cKlf-4*, and *c-Myc*, and also *cNanog*. Surprisingly, we observed that cultured PGCs also expressed all of these pluripotency genes (Fig. 4). STO feeder cells did not express any of these chicken genes (data not shown). CEFs expressed *cKlf-4* and a low level of *c-Myc*. *Klf4* and *c-myc* are expressed in many tissues during embryogenesis in mouse and rat and are not strictly markers of pluripotency alone [42,43,44]. These data show that the PGCs express many pluripotency genes in common with cES cells.

Cultured PGCs colonise the forming gonad and undergo meiosis

To validate that cultured PGCs formed functional germ cells, i.e. colonise the forming gonad and differentiate into functional gametes, we first tested the cells for their ability to migrate to the gonad. We used cultured lines of male PGCs that had been generated from a transgenic line of chickens that expressed GFP ubiquitously (GFP^+, [20]). GFP^+ PGCs were injected into the vascular system of day 3 embryos *in ovo* (stage 16 HH). Within two hours of injection, GFP^+ cells were clustered in the lateral plate mesoderm in the caudal region of the embryo (data not shown). Embryos were resealed and incubated until day 5 of development (stage 26HH). An examination of the ventral aspect of the embryo revealed that GFP^+ cells were clustered along the ventral midline of the embryo surrounding the forming genital ridges (Fig. 5A). By day 10 of development, GFP^+ cells could be seen throughout the developing gonad (n = 3 of 3, data not shown).

We extended this analysis by examining the gonads of sexually mature (16 weeks post hatch) recipient roosters. In the gonads of

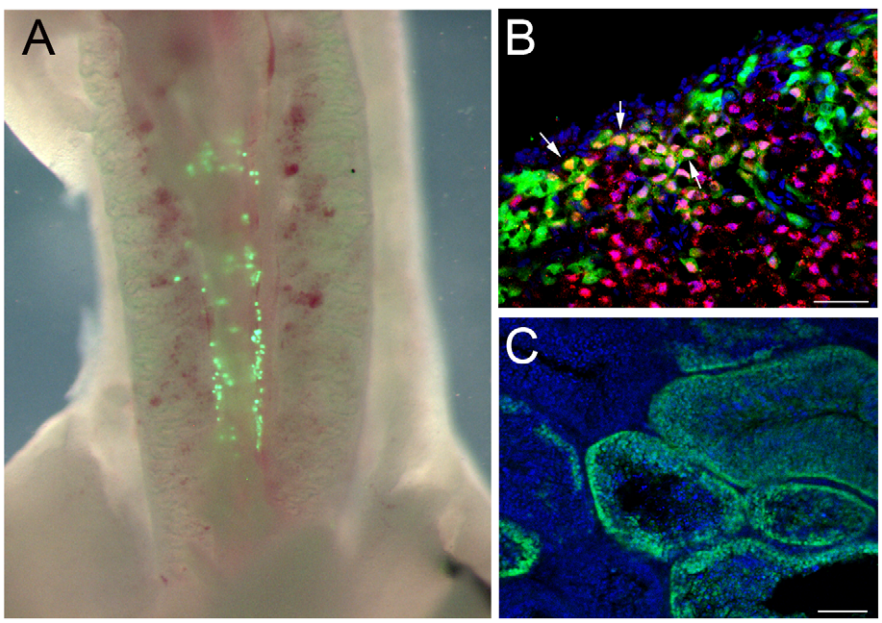

Figure 5. PGCs colonise the gonad and undergo meiosis in temporal accordance with the host embryo. A) Ventral view of a day 5 chicken embryo that was injected at stage 16 HH with GFP^+ cultured PGCs. The GFP^+ cells are found near the forming genital ridges. **B**) Section of ovary from a Day 7 hatchling immunostained for Scp3. Some GFP^+ PGCs are positive for the meiotic marker, arrows. Bar, 50ìm **C**) Section of a seminiferous tubule for 16 week old male host. GFP+ cells are present and juxtaposed to the basement membrane. Scp3, red; Blue, nuclear stain. Bar, 100μm.
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these birds, GFP^+ cells were located adjacent to the basement membrane in the seminiferous tubules (Fig. 5C, n = 3 of 3). A region of GFP^+ cells extended from the basement membrane partially toward the luminal surface of the tubule. The GFP fluorescence from the lentiviral transgene is not detectable in mature spermatids [20] so we could not determine by immunohistochemistry if the donor PGCs were forming functional spermatozoa. We next examined the fate of the male PGCs in female gonads. Sections from ovaries from recipient female hatchlings were examined for the presence of GFP^+ cells. GFP^+ cells were located in the cortex of the ovaries of these birds (Fig. 5B). We examined the expression of the meiotic marker, Scp3, to determine if the injected PGCs could undergo sex-specific differentiation in females (Fig. 5B). We observed that many of the GFP^+ cells in the ovarian cortex co-expressed Scp3 indicating that these cells were entering meiosis in accordance with the host embryo (n = 3 of 3). Thus, the cultured male PGCs were able to colonise both male and female gonads and differentiate.

Germline transmission of cultured PGCs

We next tested if the cultured PGCs were germline competent, i.e. would these cells form functional gametes and produce GFP^+ hatchlings when recipient birds were mated to wildtype birds. To increase the contribution of the donor PGCs to the host gonad we first determined if ã-irradiation would deplete the recipient embryo of endogenous PGCs. Fertile laid eggs were irradiated at selected doses of ã-irradiation, from 5–7.5 Gy, and incubated for six days. We observed that at doses above 5 Gy, embryonic development was delayed by 24 hours such that six day incubated embryos exhibited the morphological development of day 5 (stage 26HH) embryos. We carried out *in situ* hybridisation analysis using a riboprobe for *cPouV* to visualise the germ cells in the embryo (Fig. 6, top). Embryos were sectioned and *cPouV* expressing cells were counted. At doses above 5.0 Gy, germ cell number was significantly reduced (Fig. 6, bottom): at 5.0 Gy, the average germ cell number in day 5 embryos was 101. 4±23.6, at 7.0 Gy, PGC number was 70.2±27.1, at 7.5 Gy, PGC number was 13.3±4.1. Control day 5 embryos contained 2508±235 PGCs. We found that the highest dose of 7.5 Gy compromised development (50% survived to day 16 versus 64% for 7.0 Gy), so we used the lower dose of 7.0 Gy for recipient embryos.

To demonstrate that the cultured PGCs were germline competent, we injected the cells into host embryos and raised these birds to sexual maturity. We injected GFP^+ PGCs isolated from a single GFP^+ transgenic embryo (10-08-09) in FGF supplemented medium and which had been propagated for 53 days in culture. 100–500 PGCs were injected into day 3 (stage 16 HH) embryos *in ovo* that were either non-irradiated or irradiated at 7.0 Gy. Embryos were incubated until hatch. The results from two separate experiments are shown in Table 1. 26 embryos were injected and 12 embryos survived to hatch (7 males, 5 females). Of these embryos, 71% of non-irradiated (5/7) and 37% of irradiated embryos (7/19) survived. The hatchlings were raised to sexual maturity and semen samples were assayed from the roosters for the presence of the GFP transgene. All roosters contained DNA deriving from the donor PGCs in their semen at estimated frequencies from 1–30% (see materials and methods). Three roosters containing the highest estimated contribution from donor PGCs in their semen were crossed with wildtype hens and the hatched chicks were screened for GFP fluorescence. As shown in Table 1, germline transmission of the injected PGCs was observed with all three roosters. The corrected frequency of transmission was between 2–16% which correlated well with the estimated frequency of transgenic DNA in the semen samples. The number

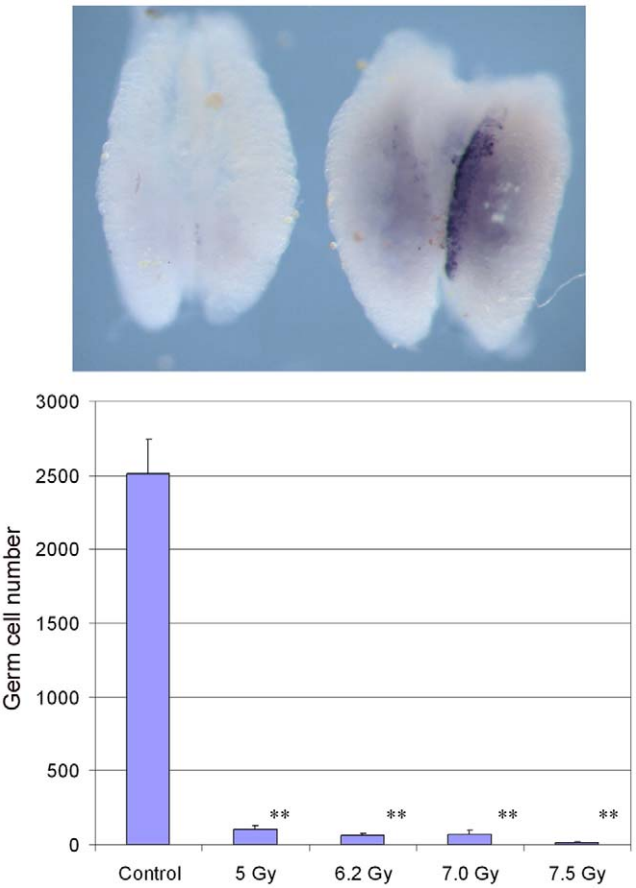

Figure 6. Y-irradiation of host embryos ablates endogenous PGCs. Top: *In situ* hybridisation with a riboprobe to *cPouV* of day 5 gonads from a control and 7.5 Gy irradiated embryo. **Bottom:** Germ cell numbers in control embryos and embryos irradiated for various times. Error bars, S.E.M. **, $p<0.01$.
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of roosters screened was too small to confirm that prior irradiation of host embryos increased the contribution of donor PGCs to the germline, although the results indicate that this may be correct. A post mortem examination of the testes (n = 7 of 7) showed that GFP^+ cells were present in all the birds. The amount of fluorescent tissue correlated well with PCR expression data.

Three recipient hens that were injected with GFP^+ PGCs (Table 1) were crossed with wild type males and embryos from these crosses were screened for GFP fluorescence. No GFP fluorescence was observed in these embryos (n = 0 of 188). The recipient hens were sacrificed and their ovaries were examined for the presence of GFP^+ cells. No GFP fluorescence was observed in the ovaries of these birds (shown = 3 of 3)). Since GFP^+ cells had been present in cortex of female birds examined at hatch (Fig. 5B, above), we conclude that the donor male PGCs were lost from the female ovary during sexual maturation. These results indicate that the donor male PGCs form functional gametes in male recipients but suggest that they are lost from the ovaries of female recipients during oogenesis.

Discussion

Characterisation of chicken PGCs

We have shown that cultured PGCs have the expected distinctive cellular morphology consisting of a large nucleus and

Table 1. Frequency of germline transmission of donor GFP+ cPGCs in host roosters.

Founder Birds♂	Eggs set	Chicks Screened (%)	% genome equivalents in semen	GFP$^+$offspring (% transmission*)
PGC 2–13	242	147 (61%)	6	2 (2.8%)
PGC 3–6	242	83 (34%)	30	7 (16.8%)
PGC 3–11	190	110 (58%)	2	1 (1.8%)
PGC 2–3	-	-	1	-
PGC 3–5	-	-	4	-
PGC 3–10	-	-	1	-
PGC 3–12	-	-	1	-
Founder Birds♀	Embryos examined	GFP$^+$ embryos		
PGC 2–2	63	0		
PGC 2–7	57	0		
PGC 3–3	68	0		

Irradiated birds are shown in bold.
*the actual transmission rate is double the observed number of GFP$^+$ chicks due to the heterozygosity of the GFP allele and meiotic reduction.
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a cluster of large vacuoles present in the cytoplasm (Fig. 3). Using a lipid stain, we demonstrated that lipid is present within some of the larger vacuoles of the cultured PGCs. This is not simply an *in vitro* culture artefact as chicken circulatory PGCs *in ovo* were also reported to contain large lipid vacuoles [29]. The lipid rich cytoplasm observed in chicken PGCs is similar to that of migratory human PGCs [45,46] but is unlike the cytoplasm of migratory PGCs in both the mouse and the pig which do not contain lipid vacuoles [47,48]. The high glycogen content and diffuse staining seen in cultured PGCs agrees with previous observations in which PAS staining was also used to identify migratory chicken PGCs [11,29,49]. Active mitochondria were found to be present throughout the cytoplasm of cultured PGCs. The cell surface marker, SSEA-1, has been shown to be present on various undifferentiated progenitor cells including ES cells and EG cells [8]. Here, SSEA-1 was also shown to be present on cultured PGCs. This is in line with previous descriptions of SSEA-1 expression on both chicken and mouse PGCs [49,50,51].

The germ cell-specific protein CVH was restricted to the cytoplasm of cultured PGCs. In Drosophila, VASA is a member of a DEAD-box RNA helicase family specific to germ cells [36]. It is indispensible for development through regulation of mRNAs such as *Nanos*. The mouse vasa homologue (MVH) was discovered to play a role in RNA processing of both mRNAs and piRNAs in germ cells and to be localised to cytoplasmic granules some of which are closely associated with mitochondria [38,52]. It will be of interest to determine if CVH displays a similarly sub-cellular localisation in cytoplasm of chicken PGCs.

The germ cell protein dead end functions to neutralize the inhibitory effects of several miRNAs allowing the expression of key genes, such as *Nanos* in PGCs [53]. In mouse *dead end homologue* mutants, a loss of PGCs and an increased susceptibility to spontaneous testicular germ cell tumour formation was observed [37]. In this work we observed that some CDH was present in the cytoplasm of cultured PGCs. This is in contrast to previous observations where CDH was described as exclusively nuclear in chicken PGCs [39]. It has been shown in zebrafish PGCs that dead end is localised predominantly to perinuclear granuoles in the cytoplasm and is thought to play a role in shuttling mRNAs between the nucleus and cytoplasm [54]. It is possible that CDH could be acting in a similar manner in chicken PGCs.

Expression of pluripotency genes in chicken PGCs

Mouse PGCs can be propagated *in vitro* for short periods before undergoing apoptosis [55]. In the presence of bFGF, SCF, or high levels of activation of the AKT signalling pathway that is downstream of the SCF receptor, mouse PGCs will de-differentiate into EG cells [5,56]. Mouse PGCs express the pluripotency markers, *Oct3/4*, *Nanog*, *Klf2*, and *Sox2*. Upon de-differentiation into EG cells, *c-Myc* and *Klf4* expression is initiated, suggesting that the expression of these additional factors may be sufficient to achieve a pluripotent state [41]. Similarly, it has been shown that porcine neonatal spermatogonial stem cells (SSCs) express low levels of *Oct3/4*, *c-Myc*, and *Sox2* and these levels and those of *Nanog* and *Klf4* increased during culture concomitant with the acquisition of pluripotency [57]. Indeed, the over-expression of c-Myc, Sox2, Klf4, and Oct3/4 transcription factors are sufficient to reprogram a somatic cell to pluripotency [58].

Here, we have shown that cultured chicken PGCs express *c-Myc*, *cSox2*, *cKlf4*, *cPouV* and *cNanog*, similar to the expression pattern seen in pluripotent cES cells (Fig. 4 and [25]). This suggests that the lineage restriction of chicken germ cells to gamete formation may not be due to the absence of pluripotency factors but may lie in epigenetic modifications of the genome or by the action of germ cell-specific proteins including CDH and CVH. It will be of interest to determine whether the expression of homologues of the pluripotency genes in other vertebrate species with early segregation of the germ cell lineages such as *Xenopus*, medaka, and zebrafish is comparable to chickens.

The *in vitro* propagation of chicken PGCs

We found that the addition of bFGF to culture medium increased the frequency of derivation of PGC cultures (Fig. 1). However, the addition of SCF, with or without bFGF, did not increase the frequency of PGC culture derivations. Although we had predicted that addition of SCF would increase the isolation of PGC cultures, as SCF is a know survival factor for mouse PGCs [31,32,33], our observations may reflect the levels of SCF in the culture media tested. BRL cells are known to secrete the growth factors LIF, SCF, and IGF-1 [59,60,61], so additional SCF may not be needed in the presence of BRL conditioned medium. As noted above, increased SCF/c-kit signalling can drive the conversion of PGCs into EG cells and inhibit PGC propagation [5,56].

We also observed that both the MEK inhibitor, PD0325901, and the PI3K inhibitor, LY294002, significantly inhibited growth of PGCs in culture. Similar results were seen for inhibition of MEK/MAPK signalling in mouse PGCs [22,35]. However, it was also reported that inhibition of PI3K signalling in mouse PGCs, using the equivalent concentration of inhibitor, had no effect on germ cell numbers [22]. This difference in experimental results could be attributed to the increased proliferation of chicken PGCs under our culture conditions in comparison to mouse PGC cultures. In our inhibitor assays, which were initiated with approximately 1000 PGCs per well, we saw a ~20-fold increase in control cell number in seven days, whereas in the mouse PGC culture experiments, control PGC number increased only 2-fold in seven days [22]. This increase in cell number could make more subtle changes in cell proliferation apparent.

These results suggest that signalling through both PI3K pathway and MAP kinase pathways are necessary for chicken PGC proliferation in culture. SCF/c-kit signalling through PI3K has also been shown to activate MAPK in haematopoietic progenitor cells [62]. Furthermore, signalling through the FGF receptor has been shown to activate PI3K in some cell types [63]. Therefore, a more detailed examination of these pathways will be needed to ascertain the specific receptor-mediated transduction pathways functioning in PGCs. An in depth understanding of the factors required for PGC proliferation in culture will form the basis of developing a defined, serum-free culture medium for chicken PGCs.

Sex-specific differences in chicken PGCs

Mouse PGCs in opposite-sex recipients have been shown to enter meiosis and differentiation in accordance with the developmental age of the recipient embryo [64]. Male PGCs in female mice will form functional oocytes [65]. Female PGCs cannot form functional gametes in the male gonad but they do undergo the initial steps of spermatogenesis in the male gonad [66]. In the chicken, cultured PGCs did not form functional gametes when transplanted into opposite-sex recipient embryos [12]. Our data support this finding. We have demonstrated that male PGCs transplanted to female recipients have entered meiosis by hatch. Subsequently, these cells may be lost from the female ovary. These results are similar to those obtained when primary male migratory PGCs (day 3, stage 14–15) were transplanted into female recipients; <1.0% of offspring of opposite-sex germline chimeras were descended from the donor PGCs [67]. Our results suggest that, in contrast to mammalian germ cell development, male chicken PGCs do enter meiosis in developmental accordance with the host ovary and may be lost during post-natal development, indicating sex-specific differences in chicken germ cells.

The work presented here confirms the long-term propagation of primordial germ cells from chicken embryos and their competence to contribute to the germline of recipient birds. These cells will be a valuable tool for transgenic technology with both research and industrial applications and will be useful to study the genetic pathways involved in germ cell proliferation, migration, and determination.

Supporting Information

Figure S1 Chicken ES cells contribute to the three germ layers of the developing embryo. A) Day 8 embryo that was injected with GFP^+ cES cells at the laid egg stage. **B**) GFP^+ cES cells after three weeks in culture. **C**) Transverse section of the neural tube showing GFP^+ neurons. **D**) Longitudinal section of the forming limb. GFP^+ cells are in the mesoderm surrounding the forming nerve tracts **E**) Transverse section of the intestine at the level of the liver demonstrating GFP^+ cell contribution to the endodermal cell layer. Nuclear stain, blue; Tuj III neuronal marker, red. Bar, 200m.
(TIF)

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Author Contributions

Conceived and designed the experiments: JM JDG HMS MJM. Performed the experiments: JM LT JDG MJM. Analyzed the data: JM JDG HMS LT MJM. Contributed reagents/materials/analysis tools: MJM. Wrote the paper: JM JDG HMS MJM.

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